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Cloning and functional characterization of the *WdSTUA* and *WdPACC* genes of *Wangiella dermatitidis*

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Dedication

To my grandma, my parents and my husband, Zhenyu

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Cloning and functional characterization of the *WdSTUA* and *WdPACC* genes of *Wangiella dermatitidis*

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To study the function of WdStuAp and WdPacCp in *Wangiella dermatitidis,* a black, polymorphic fungal pathogen of humans with yeast phase predominance, *WdSTUA* and *WdPACC* were cloned, sequenced, disrupted and expressed. WdStuAp was most similar to the APSES proteins of *Aspergillus* species and its APSES DNA-binding domain was located in its N-terminal half. Deletion of *WdSTUA* in *W. dermatitidis* induced convoluted instead of normal smooth colony surface growth on the rich, yeast maintenance agar medium, YPDA, at 37°C. Additionally, deletion of *WdSTUA* repressed aerial hyphal growth, conidiation and invasive hyphal growth on the nitrogen poor, hyphae-inducing agar medium, PDA, at 25°C. Ectopic expression of *WdSTUA* repressed the convoluted colony surface growth on YPDA at 37°C, and also strongly repressed hyphal growth on PDA at 25°C and 37°C. Expression of *WdSTUA* in *S. cerevisiae* induced pseudohyphal growth on the nitrogen poor medium. WdPacCp was also most similar to the PacCp proteins of *Aspergillus* species. Three zinc finger DNA-

binding motifs were at the N-terminus, and the C-terminus had the signaling protease cleavage site. *WdPACC* was more expressed at neutral-alkaline pH than at acidic pH. Truncation of the coding sequence for about 40 residues upstream of the conserved processing protease cleavage site of WdPacCp affected growth on YPDA, increased sensitivity to Na^+ stress, decreased growth level at neutral-alkaline pH, and repressed hyphal growth on PDA at 25°C. Truncation of the coding sequence for the conserved signaling protease box of WdPacCp impaired growth and reduced RNA expression of class II chitin synthase gene *WdCHS1* at acidic pH, and activated hyphal growth on PDA. My results suggested that WdStuAp and WdPacCp play important roles in yeast-hyphal transitions in *W. dermatitidis,* and that WdPacCp is particularly important for *W. dermatitidis* to adapt to different ambient pH conditions.

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Chapter 1 General introduction

1.1 Relationship of morphotypes to fungal pathogenesis

The occurrence of fungal infections is increasing, especially in immuno-compromised people. In many cases, fungal infections even lead to mortality. Treatment of fungal infections, particularly systematic fungal infections, is difficult because host and fungal cells have genetic similarities, as they both are eukaryotic. The drugs currently used in therapy often display pharmacological problems, thus limiting their clinical applications. This has motivated the scientific community to explore the pathogenic fungi for alternative virulence determinants that might be used as new antifungal targets. The goal of such research is to find therapeutic drugs that alter these targets and thus limit infections (Alonso-Monge et al., 2003).

Among the more than 100,000 identified fungi, only a relatively few are pathogenic (Rooney and Klein, 2002). Of these, a relatively large percentage can grow in different forms called morphotypes, such as hyphae, yeast, spherules and sclerotic forms, and more often than not the growth form correlates with pathogenesis. For example, the dimorphic pathogens *Penicillium marneffei*, *Histoplasma capsulatum, Blastomyces dermatitidis,* and *Sprothrix schenckii* grow as molds (hyphal conidiogenous fungi) at an ambient temperature of 25^{o}C and as yeast at the infection temperature of 37^{o}C. Many hypotheses have been put forth to explain this phenomenon, but none are totally satisfactory.

Among them, one suggests that the yeast morphotype may more efficiently facilitate replication than hyphae and disseminate more efficiently in body fluids and host cells, such as macrophages, and thus facilitate survival. However, the most common opportunistic fungal pathogen of humans, the dimorphic, but nonconidiogenous yeast *Candida albicans*, is found to invade the tissues with hyphae. Apparently, different pathogenic fungi have specific infection advantages, among which are the abilities of their parasitic, tissue-phases to respond to a variety of environmental conditions that can trigger a switch from one morphotype to another. The resulting new morphotype then may be better able to survive in the different tissues of the human body. The fact that so many pathogenic fungi have the ability to switch between two or more different growth forms has prompted medical mycologists for years to consider that property to be a virulence determinant (Alonso-Monge et al., 2003; Rooney and Klein, 2002). This in turn has promoted numerous studies directed at enhancing our understanding the regulatory mechanisms controlling the morphotype transitions associated with the various fungal pathogens of humans. An overview of some findings related to a few of those pathogenic fungi follows.

In *P. marneffei*, the main agent of HIV/AIDS-related mycosis in Southeast Asia, the developmental transcription factor AbaAp regulates conidiation and dimorphic transition between yeast and hyphae. Deletion of *ABAA* eliminates conidium (asexual spore) formation formation at 25^{o}C and impairs hyphae-to-yeast transitions at 37^{o}C, producing yeast cells with multiple nuclei. In addition, overexpression of *ABAA* causes the formation of abnormal cells with multiple nuclei. However, the virulence of the *ABAA*

deletion strain has yet to be tested (Borneman et al., 2000). In the agent of histoplasmosis, *H. capsulatum,* the gene *CBP* (calcium-binding protein) encodes a Ca^{++}-binding protein that is secreted by the parasitic yeast phase at 37°C but not by the saprophytic filamentous phase at 25°C. In addition, *CBP* deletion strains cannot grow under Ca^{++}-limiting conditions, cannot destroy macrophages, and cannot multiply in a mouse model (Sebghati et al., 2000). Furthermore, the α- 1, 3 glucan located in the outermost layer of this pathogen's cell wall allows it to evade host immune reactions to its cell wall β-1, 3 glucan: deletion of β-1, 3 glucan receptor also reduces virulence (Rappleye et al., 2007). In the blastomycosis agent *B. dermatitidis,* BAD1 (*Blastomyces* adhesin) acts as a 37°C yeast phase-specific adhesive protein that is located at the cell surface and is also secreted. During infection, BAD1 modulates the immune reaction, and deletion of the *BAD1* gene reduces virulence (Brandhorst and Klein, 2000; Finkel-Jimenez et al., 2001; Hogan et al., 1995). In contrast to these agents, which undergo yeast-hyphal transitions, the conidiogenous agent of coccidioidomycosis, *C. immitis,* exhibits conidial and hyphal trasitions to spherules instead of yeast at 37°C both *in vivo* and *in vitro*. In this pathogen, the *SOWgp* gene (spherule outer-wall glycoprotein) encodes a spherule outer-wall antigen that is required for virulence (Hung et al., 2002). The metalloproteinase Mep1p removes SOWgp from the endospores (Hung et al., 2005). Also a urease produced by the sherules is required for creating an alkaline environment for infections (Mirbod-Donovan et al., 2006).

Although *Aspergillus* species are not considered to be dimorphic, because they grow vegetatively both *in vitro* and *in vivo* by producing only hyphae, they do carry out

morphological transitions from conidia to hyphae to conidia. With these pathogens, inhalation of conidia into the lungs of humans is the main route of infection leading to aspergillosis. Colonization of the lungs follows conidial germination. Depending on the immunological status of the host, the hyphae that result then may or may not become invasive through additional hyphal growth and hyphal branching. In *A. nidulans*, as in most other aspergilli, conidiation is induced by exposure to air and nutrient limitations, with conidiophores developing by differentiation of the aerial hyphae. In this mold, the process is complex with the conidiophores first generating vesicles, then metulae and phialids on the vesicles, and finally conidia. To complete this process, the transcription factors BrlAp, AbaAp, and WetAp are required for the formation of the conidia. Interestingly, the conidiophores of the *BRLA* deletion strain only produce apical vesicles. The *BRLA* deletion strain, which is characterized as the bristle phenotype, also produces longer (20–30 X) conidiophores than the wild type. In contrast, an *ABAA* deletion strain, which is characterized as the abacus phenotype, produces conidiophores with both vesicles and metulae, but without phialides and conidia (Clutterbuck, 1969). Both deletion and overexpression of *ABAA* activate the expression of *BRLA*, which indicates that *ABAA* has both activation and repression functions (Aguirre, 1993). Finally, the *WETA* deletion strain, which is characterized as the white phenotype due to absence of conidia, produces conidiophores with vesicles, metulae, and phialides, but without conidia (Sewall et al., 1990). Two additional transcription factors, StuAp and MedAp, act as developmental modifiers in this process. The deletion of *STUA* produces strains with conidiophores, vesicles, and conidia but not metulae and phialides. Studies of *BRLA*:*lacZ* and the *ABAA*:*lacZ* translational fusions show they are mis-located in the *STUA* deletion

strain (Miller et al., 1991; Miller et al., 1992). In contrast, the *MEDA* deletion strain produces conidiophores, vesicles and conidia, but with multiple layers of metulae and phialides (Clutterbuck, 1969). *MEDA* is required for the temporal expression of *BRLA* and the normal level of expression of *ABAA* (Busby et al., 1996).

Filamentous growth and transitions between yeast and hyphae in *C. albicans* can result from processing a variety different environmental signals, such as those resulting from culture in serum, at neutral pH, under nitrogen starvation, and at high temperature. The finding that nonfilamentous *C. albicans* strains derived by *EFG1* and *CPH1* double deletion is avirulent (Lo et al., 1997) led to the discovery of many signal transduction pathways that regulate the hyphal-yeast transition. Among these are the MAPK-Cph1p pathway that includes Ras1p (G protein), Cst20p (p21-activated kinase), Hst7p (MAPKK), Cek1p (MAPK), and Cph1p (transcription factor), which regulate mating and hyphal growth and a cAMP-dependent protein kinase A (PKA)-Efg1p pathway that includes Gpr1p, Gpa2p and Ras1p (G protein), Bcy1p and Cap1p (adenylate cyclase), cAMP, Tpk1p and Tpk2p (PKA), and Efg1p (transcription factor), which are involved in the regulation of hyphal growth. In addition, the Tup1p transcription repressor complex inhibits filamentous growth, Rim101p, a pH pathway transcription factor, regulates the pH response gene and activates hypha-specific genes and the transcription factors Czf1p, Tec1p (AbaAp ortholog), and Cph2p are known to control hyphal growth (Liu, 2001; Whiteway and Oberholzer, 2004).

Alsp (agglutinin-like sequence) family proteins are glycosylphosphatidylinositol- (GPI-) cell wall proteins (CWP) that have three domains: a conserved N terminal domain, a central tandem repeat domain, and a C terminal domain with divergent sequences. The N-terminus and C-terminus also contain a conserved secretary signal sequence and a conserved GPI addition site, respectively (Hoyer et al., 1998b). In *C. albicans* at least two *ALS* genes, *ALS3* and *ALS8*, are induced in hyphal development (Hoyer et al., 1998a; Leng et al., 2001). Alsp family proteins are also required for filamentous growth, to confer adhesion to host surfaces, and are involved in pathogenesis (Fu et al., 2002; Hoyer, 2001). Transformation of several *ALS* genes into *S. cerevisiae* makes cells adhere to extra-cellular matrix (ECM) proteins, endothelial and epithelial cells (Fu et al., 1998; Gaur and Klotz, 1997; Gaur et al., 2002; Sheppard et al., 2004). The hyphal wall protein (Hwplp) is also a GPI-CWP (Staab and Sundstrom, 1998). The N terminal region of Hwp1p is similar to the substrates of mammalian transglutaminases, and Hwp1p has been shown to function as a transglutaminase substrate. Transglutaminases crosslink epithelial cell proteins, so that Hwp1p is also crosslinked by transglutaminases to these proteins, forming stable complexes. Deletion of *HWP1* reduces the attachment of *C. albicans* to epithelial cells and reduces systemic infections in mice (Staab et al., 1999) and *HWP1* is induced during hyphal growth and biofilm formation (Nobile and Mitchell, 2006; Sohn et al., 2003). Furthermore, the β-1, 3-glucanosyltransglycosylases Phr1p (pH-regulated) and Phr2p are differentially expressed at alkaline and acidic pH, respectively, and are required for morphogenesis and virulence (Ghannoum et al., 1995; Muhlschlegel and Fonzi, 1997; Saporito-Irwin et al., 1995).

1.2 *Wangiella dermatitidis* as a model fungal system

Wangiella (*Exophiala*) *dermatitidis* is a unique fungal pathogen that was first identified during the study of a patient's skin lesion. It is saprophytic in soil, on decaying timber, and in other plant materials. In humans, *W. dermatitidis* causes superficial, cutaneous, subcutaneous, and systemic pheohyphomycosis (Matsumoto et al., 1994). Systemic pheohyphomycosis caused by *W. dermatitidis* includes respiratory, intestinal, cardiac, and cerebral infections (de Hoog et al., 2005; Mukaino et al., 2006; Tseng et al., 2005). Although *W. dermatitidis* is asexual, and thus can't be classified by traditional morphological methods, numerous molecular phylogenetic sequence analyses indicate that it belongs to Ascomycota phylum, the Pezizomycotina subphylum, and the Chaetothyriomycetes class. Thus *W. dermatitidis* and *S. cerevisiae* are classified in the same phylum, but in different subphyla, whereas the *W. dermatitidis* and *Aspergillus* species are classified in the same subphylum.

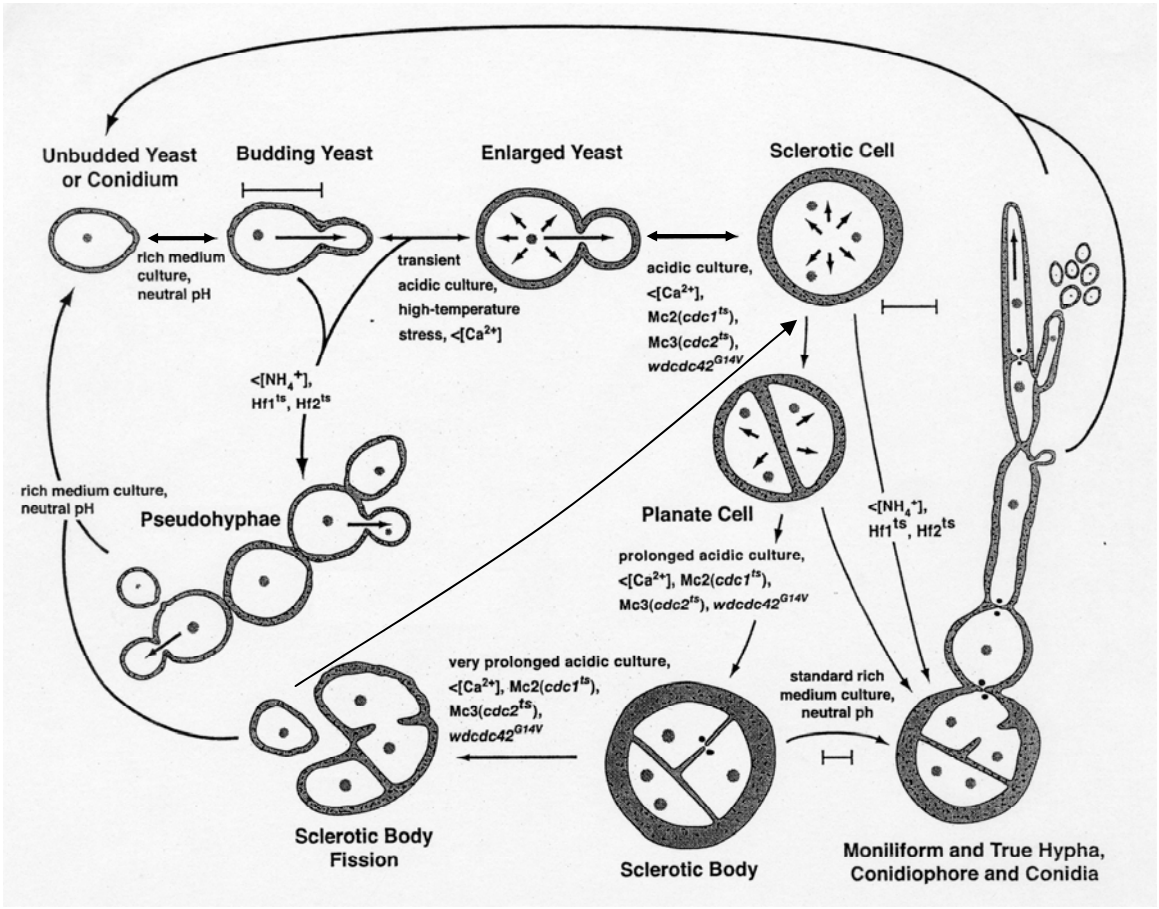

FIGURE 1.1. MORPHOTYPES OF AND FACTORS AFFECTING MORPHOLOGICAL TRANSITIONS IN *W. DERMATITIDIS* (adapted from Szaniszlo, 2006).

The predominant morphotype of *W. dermatitidis* is a budding yeast cell, although it can also produce pseudohyphae, moniliform hyphae, true hyphae, sclerotic forms, and conidia (Figure 1.1). At the light microscope level, pseudohyphae and moniliform hyphae appear very similar, but at the transmission electron microcope (TEM) level they are seen to be very different, with the former being chains of yeast cells that fail to separate and the moniliform hyphae like true hyphae being distinguished by septa with central pores and adjacent Woronin bodies (Oujezdsky et al., 1973). However, moniliform hyphae like pseudohyphae have constrictions at septal regions, whereas true hyphae have parallel side walls. In stark contrast, sclerotic forms are isotropically enlarged and can become divided by transverse and intersecting septa, which thus produces the multicellular morphotype. Finally, the conidia of *W. dermatitidis* are produced from the conidiophores that are developed from the aerial hyphae. Yeast, pseudohyphae, moniliform hyphae, true hyphae, and sclerotic morphotypes are all found in infected tissues. Although the conidia of *W. dermatitidis* can be inhaled to cause infections, most infections are initiated by the traumatic implantation of the fungus into cutaneous and subcutaneous tissue.

Yeast-to-mold transition in *W. dermatitidis* has been critically monitored by transmission electron microcope (Oujezdsky et al., 1973). On agar media, a yeast cell first becomes an isotropically enlarged, thick-walled cell that contains many lipid bodies. This cell is then competent to produce moniliform hyphae, which in turn may then generate true hyphae (Oujezdsky et al., 1973). Hyphal growth in *W. dermatitidis* can also be induced under nitrogen limitation conditions and in temperature-sensitive, so called hyphal-form

mutants (Hf1 and Hf2) (Szaniszlo, 2006; Wang and Szaniszlo, 2000; Ye and Szaniszlo, 2000) by shift from 25°C to 37°C. On the otherhand, sclerotic form growth is induced at pH 2.5, by calcium limitation at pH 6.5, and in temperature-sensitive multicellular-form mutants (Mc 2/*cdc1* and Mc3/*cdc2*) (Cooper and Szaniszlo, 1993; Karuppayil and Szaniszlo, 1997; Szaniszlo, 2006; Szaniszlo et al., 1976). Also in *W. dermatitidis*, *WdCDC42* encodes a Rho/Rac member of small GTP-binding proteins, and the dominant active mutation $WdCDC42^{G14V}$ activates sclerotic form growth at 37°C and represses the filamentous growth of temperature-sensitive hyphal-form mutant Hf1 (Ye and Szaniszlo, 2000).

The pathway leading to melanin biosynthesis in *W. dermatitidis* is from acetate or malonate to 1, 3, 6, 8- THN (tetrahydroxynaphthalene) that is reduced to scytalone that in turn is then dehydrated to 1, 3, 8- THN (trihydroxynaphthalene), followed by its reduction to vermelone and dehydration of vermelone to produce 1, 8- DHN (dihydroxynaphthalene). Finally, by oxidation, 1, 8- DHN is polymerized to 1, 8- DHN-melanin in the cell wall. By providing downstream products, mutants with upstream gene mutations produce and deposit 1, 8- DHN- melanin in their cell walls (Cooper, 1997). Transmission electron microscopy shows that the outside part of the cell wall of wild-type *W. dermatitidis* is opaque, in contrast to that of the white mutants, probably because of the deposition of melanin in that region (Wheeler and Stipanovic, 1985). Melanin is thought to protect cells against the oxidative stresses encountered in the environment, and white strains have less virulence in the infection of mice and are more easily killed by nertrophils (Dixon, 1991; Feng et al., 2001). *WdPKS1* encodes a type I polyketide

synthase that has been thought to be the enzyme that catalyzes the first cyclized product in the pathway 1, 3, 6, 8- THN, and deletion of *WdPKS1* has been shown to make strains become white (Feng et al., 2001). However, recently the gene *WdYG1* has been cloned, and its disruption suggest that the first product of WdPks1p may in fact be a heptaketide which WdYg1p converts to 1, 3, 6, 8- THN (D. Abramczyk, M. Wheeler and P.J. Szaniszlo, unpublished data).

Chitin synthases, which are located in the plama membrane and deposit chitin to the cell wall, have a conserved chitin synthase domain and cross-membrane motifs. In *W. dermatitidis*, five kinds of chitin synthases have been reported, in contrast to the three kinds in *S. cerevisiae*. Chitin synthases can be divided into two groups and at least five classes. Group I is divided into three classes—I, II, and III—and ScChs1p and ScChs2p belong to classes I and II, respectively. Group II is divided into two classes—IV and V—and ScChs3p is included in class IV. The five reported *W. dermatitidis* chitin synthases belong to five classes: class II, WdChs1p (Zheng et al., 2006); class I, WdChs2p (Wang et al., 2001); class III, WdChs3p (Wang and Szaniszlo, 2000); class IV, WdChs4p (Wang et al., 1999); and class V, WdChs5p (Liu et al., 2004). Strains with mutations in one or more of the genes encoding these proteins have the following characteristics. The mutant *wdchs1*Δ is hyperpigmented, and has enlarged, multinucleate, abnormal cells at 25^{o}C and 37^{o}C (Zheng et al., 2006). In contrast, *wdchs2*Δ shows no morphological defects. WdChs1p and WdChs2p may have some overlapping functions, which is supported by results from the double disruption of *WdCHS1* and *WdCHS2*. For example, the *wdchs1*Δ*wdchs2*Δ mutant dies at 37^{o}C and grows weekly at 25^{o}C, and in turn it has no

detectable virulence in a mouse model of acute infection (Zheng et al., 2006). Whereas *wdchs3*Δ also shows no morphological defects. *wdchs2*Δ*wdchs3*Δ mutants, but not the *wdchs1*Δ*wdchs3*Δ mutants, have reduced virulence in a mouse model (Wang et al., 2001). The phenotypes of triple mutants *wdchs1*Δ*wdchs2*Δ*wdchs3*Δ and *wdchs1*Δ*wdchs4*Δ*wdchs3*Δ are similar to those of *wdchs1*Δ*wdchs2*Δ and *wdchs1*Δ*wdchs4*Δ, with the latter having the hybrid phenotype of the *wdchs1*Δ and *wdchs4*Δ cells: *wdchs4*Δ cells are hyperpigmented, tend to clump and exhibit abnormal budding, particularly at 37^{o}C (Wang et al., 1999). In *S. cerevisiae,* a *scchs2*Δ*scchs3*Δ mutant (defective in both its class II and class IV chitin synthases) is not viable; however, *wdchs1*Δ*wdchs4*Δ (defective in the same two types of chitin synthases) is viable, with the phenotype of the *wdchs1*Δ*wdchs4*Δ mutant being a combination of the phenotypes of *wdchs1*Δ and *wdchs4*Δ, suggesting that other *W. dermatitidis* chitin synthases compensate for the disruption of *WdCHS1* and *WdCHS4.* The *wdchs1*Δ*wdchs3*Δ*wdchs4*Δ triple mutant retains the wild type virulence in a mouse model (Szaniszlo, 2002). At 37°C in late-log- or early stationary-phase, the *wdchs5*Δ mutant is darker than the wild type and cells of *wdchs5*Δ blow out or otherwise slowly die, so that their virulence in a mouse model is decreased by the disruption of *WdCHS5* (Liu et al., 2004).

While a number of genes of *W. dermatitidis* have been shown to encode proteins that when defective affect its morphology, nothing is known about how transcription factors control the morphogenesis of this fungal pathogen of humans. Therefore, the purpose of this study was to investigate the importance of the two transcription factor genes *WdSTUA* and *WdPACC* and particularly their effects on cellular development and

differentiation. The study of *WdSTUA* and *WdPACC* would significantly broaden our knowledge of these transcription factors in fungi in general, and particularly in *W. dermatitidis,* a filamentous conidiogenous fungus with a yeast phase predominance.

Chapter 2 General materials and methods

2.1 Media and growth conditions

Wangiella dermatitidis strain 8656 (ATCC34100) was the wild-type strain used in this work. Routine culture of *W. dermatitidis* was on YPD agar (YPDA; 1% yeast extract, 2% peptone, 2% dextrose, 2% agar) and in YPD broth (YPDB) at 25^{o}C as described previously (Liu et al., 2004). For the transformations of *W. dermatitidis*, YPDA containing 50 μg/ml hygromycin B (Invitrogen, Carlsbad, CA) was used to select resistant transformants that contained the hygromycin phosphotransferase gene (*hph*, GenBank accession number AF013601), or SD agar (0.17% yeast nitrogen base w/o amino acid and ammonium sulfate, 0.5% ammonium sulfate, 2% dextrose, 2% agar) containing 20 μg/ml chlorimuron ethyl (Fisher, Pittsburgh, PA) was used to select the resistant transformants that contained the acetolactate synthase gene [*sur* (sulfonylurea resistant), GenBank accession number AY142483]. To detect or induce filamentous growth, potato dextrose agar (PDA; Difco Scientific, Detriot, Mich.), potato dextrose broth (PDB; Difco Scientific, Detriot, Mich.), corn meal agar (CMA; Difco Scientific), corn meal dextrose agar (CMDA; Difco Scientific), synthetic low-ammonium dextrose agar [SLADA; 0.17% yeast nitrogen base w/o amino acid and ammonium sulfate, 50 μM ammonium sulfate, 2% dextrose, 2% agar;], Sabouraud dextrose agar (SDA; Difco Scientific), yeast peptone maltose agar [YPMA; 1% yeast extract, 2% peptone, 2% maltose, 2% agar; (Ye et al., 1999; Ye and Szaniszlo, 2000)] and yeast peptone sucrose

agar [YPSA; 1% yeast extract, 2% peptone, 2% sucrose, 2% agar; (Brown et al., 1999)] were used. For the induction of sclerotic forms, 25°C YPDB cultured *W. dermatitidis* cells were washed and inoculated into modified Czapek Dox broth [MCDB; 3.5% Czapek Dox, 0.1% yeast extract, pH adjusted with HCl; (Wang and Szaniszlo, 2000)] inoculated with 10^7 cells/ml and shaken vigorously at 25°C for several days. For storage, *W. dermatitidis* stationary phase cells (0.5 ml) were mixed with 0.15 ml 65% glycerol to reach 15% final concentration and stored at -80^{o}C. The restrictive temperature for the ts hyphal form mutant Hf1 was 37^{o}C.

E. coli strain XL1-blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*qZΔ*M15* Tn*10* (Tet^r)]; Stratagene, La Jolla, CA) was used for cloning and plasmid preparation, and *E. coli* strain DH10B (F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139Δ(*ara, leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G; Invitrogen) was used for construction of the partial genomic library. *E. coli* was grown in Luria-Bertani medium (LB; 1% tryptone, 1% NaCl, 0.5% yeast extract) at 37^{o}C. LB containing 100 μg/ml ampicilline or LB containing 20 μg/ml chloramphenicol was used when the plasmid was introduced into the *E. coli* cells. For storage, *E. coli* stationary-phase cells (0.5 ml) were mixed with 0.5 ml 65% glycerol to reach the final concentration of ~30% and stored at -80^{o}C.

2.2 Reagents and equipment

Primers were synthesized by Invitrogen (Carlsbad, Calif.). Enzymes were usually perchased from Promega (Madison, WI). Reagents were usually purchased from Sigma (Saint Louis, MO) and Fisher (Pittsburg, PA). The QIAprep miniprep kit (Qiagen, Chatsworth, CA) was used for the preparation of plasmid DNA. The QIAquick gel extraction kit (Qiagen) and the MinElute PCR purification kit (Qiagen) were used to purify DNA. The RNeasy Mini kit (Qiagen) was for the isolation of total RNA. The pGEM-T easy vector kit (Promega) was for the ligation of PCR products. The DECAprime II random priming DNA labeling kit (Ambion, Austin, TX) was used to label probes. Isotopes were purchased from Dupont NEN (Boston, MA). Nytran N nylon membranes were from Schleicher & Schuell (Keene, NH). PCR amplifications were carried out with a 2720 thermal cycler (Applied Biosystems, Foster City, CA). A pulser transformation apparatus (Bio-Rad, Hercules, CA) was used for electroporation. UV crosslinking was performed in CL-100 Ultraviolet Crosslinker (Ultra-Violet Products, San Gabriel, CA). Hybridization was carried out in Personal Hyb Techne hybridizer HB-2D (Stratagene).

2.3 Extraction of genomic DNA

To obtain genomic DNA, stationary-phase cells of *W. dermatitidis* cultured in YPDB at 25^{o}C (5 ml) were collected by centrifugation, washed with deionized water and resuspended in 200 µl breaking buffer (2% Trition X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl, 1 mM EDTA, pH 8.0), to which 200 µl glass beads (0.5 mm glass beads, Biospect Products, Bartelsville, Okla.) and 200 µl phenol/chloroform (Ambion) were

added. The mixture was then vortexed on Multi-Tube Vortexer (VWR) at highest speed for 5 min, after which 200 µl TE (pH 8.0) was added. Following centrifugation, the supernatant was precipitated with 1/10 volume of 3M NaOAc (pH 5.2) and 2 volumes of 100% ethanol. Finally, the DNA pellet was washed with 70% ethanol, dried and dissolved in 30 µl Tris-Cl pH 8.0 (~5 µg genomic DNA), and treated with 7.5 µl of 1 mg/ml DNase-free RNase A to remove coprecipitated RNA.

2.4 Preparation of ^{32}P-DNA probes by a random priming method

The DECA prime II DNA labeling kit (Ambion) was used to label probes. Approximately 25 ng of gel-purified DNA fragments (QIAquick Gel Extraction Kit) in 2.5 µl 10x Decamer solution (contains random decamer oligos) were denatured at 100^{o}C for 3-5 min and then chilled on ice for 2 min. Subsequently, 5 µl of 5x buffer (-dATP), 1 µl of exonuclease-free Klenow and 3 µl α-^{32}P dATP (3000Ci/mmol, 10 mCi/ml, Dupont NEN) were added and the resulting mixture incubated at 37^{o}C for 30 min. After the reaction was stopped by adding 75 µl of 30 mM EDTA, the labeled probe was separated from the incorporated nucleotides by spin through a Sephadex G50 column (Sigma).

2.5 Southern analysis

Prior to Southern analysis, the *W. dermatitidis* genomic DNA (1 µg) was digested overnight with a restriction endonuclease and then subjected to electrophoresis in a 0.8% agarose gel. After the DNA was denatured by soaking and moderately shaking the gel in

0.5 M NaOH, 1.5 M NaCl for 1 h, and rinsing several times in dH_2O, the agarose gel was neutralized by soaking and moderately shaking in 1 M Tris-Cl pH 7.4, 1.5 M NaCl for 20 min twice. Using upward capillary transfer with 10x SSC (1.5 M NaCl, 0.15 M sodium citrate), the DNA in the gel was transferred overnight to a Nytran N nylon membrane (Schleicher & Schuell). After the transfer, the nylon membrane was rinsed in 2x SSC, air dried on a Whatman 3MM filter paper and then UV crosslinked (CL-100 Ultraviolet Crosslinker). The DNA-bound membrane was prehybridized in PerfectHyb Plus hybridization buffer (Sigma) in a hybridization oven (Personal Hyb Techne hybridizer HB-2D, Stratagene) for 30 min at 68^{o}C. After the probe was heated to 100^{o}C for 10 min, it was immediately placed on ice for 2 min, and then added to the hybridization tube to hybridize for 3 h at 68^{o}C. After the hybridization, the membrane was washed in low stringency wash buffer (2x SSC, 0.1% SDS) for 5 min at room temperature, and then high stringency wash buffer (0.5x SSC, 0.1% SDS) for 20 min at 68^{o}C twice, followed by exposure to a phosphorimager screen. The radioactive signals were finally detected by phosphorimager scanning (Bio-Rad, Hercules, Calif.).

2.6 Screens of the *W. dermatitidis* cosmid genomic library

For titrating a cosmid genomic library of *W. dermatitidis* (Feng et al., 2001), dilutions were plated on LB agar containing 20 μg/ml chloramphenicol. Next, 10^4 cells were spread on three 15 cm large plates and incubated at 37^{o}C for 12 h. Nylon membranes were then placed over the colonies that developed, their positions were marked with a needle, and the colonies were transferred to Whatman 3MM filter paper that was

saturated subsequently first with 0.5 M NaOH for 7 min, followed by 1 M Tris-Cl pH 7.4 for 2 min twice, then 0.5 M Tris-Cl pH 7.4 and 1 M NaCl for 4 min, and finally with 2x SSC for 4 min. The procedures for the UV crosslinking, prehybridization, hybridization, washing, exposure and radioactive signal detection were subsequently carried out similarly to those used for the Southern analysis.

2.7 Northern analysis

Total RNA was isolated with the RNeasy kit (Qiagen). After the *W. dermatitidis* cells were ground thoroughly in liquid nitrogen with a mortar and pestle to break cells, the frozen fine powders were transferred to the guanidine isothiocyanate-containing buffer RLT. The resulting lysate was then spun through a QIAshredder to remove cell debris and to homogenize the lysate. Next the supernatant was mixed with ethanol, then applied to an RNeasy spin column, which was subsequently washed with RW1 and RPE and eluted with RNase-free H_2O. The final concentration of RNA was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Del.). The quality of RNA was estimated by electrophoresis of 2 μg RNA in an RNA gel. For the Northern blotting itself, 10 μg of the RNA was mixed in 50% formamide, 1x MOPS running buffer [20 mM MOPS (3-(N-morpholino)-propanesulfonic acid), 5 mM sodium acetate and 1 mM EDTA, adjusted to pH 7 with 10 M NaOH], 2 M formaldehyde, 0.1% bromophenol blue and 0.05 mg/ml ethidium bromide, and the mixture was incubated at 65^{o}C for 5 min, followed by fast chilling on ice for 2 min. Electrophoresis of the samples was at ~5V/cm in a 1% agarose gel containing 2 M formaldehyde, with 1x MOPS

running buffer. A 0.24-9.5 kb RNA ladder (Invitrogen) was run simultaneously to provide size markers. After electrophoresis, RNA gel was rinsed with deionized water, photographed, and then soaked in 10x SSC for 10 min. The procedures for membrane transfer, UV crosslinking, prehybridization, hybridization, washing, exposure and radioactive signal detection were similar to those used for the Southern analysis.

2.8 PCR amplifications

PCR was usually carried out with 2.5 µl of 10x PCR buffer (15 mM Mg^{++}, 500 mM KCl, 100 mM Tris-HCl, pH 9), 0.5 µl of 10mM dNTP, 1 µl of 10 µM primers, 0.2 µl of Taq DNA polymerase (5U/µl, Fisher) and template DNA mixed in a final volume of 25 µl. The PCR reaction conditions were as follows: 5 min at 94°C for premelting; 30 cycles of 30 sec at 94°C for denaturation, 30 sec at 55°C for annealing, and 1 min at 72°C for extension; 7 min at 72°C for completion of the extension. The product of PCR was detected by agarose gel electrophoresis.

2.9 RT-PCR

Total RNA was treated with RQ1 RNase-Free DNase (Promega). RT-PCR was carried out with the One-step RT-PCR kit (Qiagen). RT-PCR was performed with 5 µl of 5x PCR buffer (contains Tris·Cl, KCl, $(NH_4)_2SO_4$, 12.5 mM $MgCl_2$, DTT; pH 8.7), 1 µl of 10mM dNTP, 1.5 µl 10 µM primers, 1 µl of RT-PCR enzyme mix (contains Omniscript™ Reverse Transcriptase, Sensiscript™ Reverse Transcriptase, and

HotStarTaq® DNA Polymerase) and 1 μg total RNA mixed in a final volume of 25 μl. The RT-PCR reaction conditions were as follows: 30 min at 50°C for reverse transcription and 15 min at 95°C for initial PCR activation; 35 cycles of 30 sec at 94°C for denaturation, 30 sec at 55°C for annealing, and 1 min at 72°C for extension; 10 min at 72°C for completion of the extension. The product of RT-PCR was detected by agarose gel electrophoresis.

2.10 Sequencing and computer analysis

DNA sequencing was performed by the Core Facility of Institute of Cellular and Molecular Biology, University of Texas at Austin, using Big Dye technology (Applied Biosystems). NCBI databases were searched with BLAST programs (www.ncbi.nlm.nih.gov/BLAST/). Protein structure was analyzed using ExPASy tools (expasy.org/tools/). Introns were predicted by GENSCAN (genes.mit.edu/GENSCAN.html). Multiple alignment and phylogram were done by ClustalW (www.ebi.ac.uk/clustalw/).

2.11 Transformations of *E. coli* and *W. dermatitidis*

To make *E. coli* cells competent for transformations, cells from an overnight culture (8 ml) were inoculated to 200-ml LB broth, shaken vigorously at 37°C for ~1.5 to 2.5 h until the OD_{595} reached ~0.5, and then pelleted by centrifugation. After the supernatant was discarded, the pellet was then vortexed in 30 ml ice cold TFBI buffer (30 mM KOAc, 100

mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, and 15% glycerol, pH 5.8, filter sterilized) to resuspend the pellet. This mixture was then placed on ice for 2 h, centrifuged, after which the supernatant was again discarded. Once the pellet was carefully resuspended in 5 ml ice cold TFBII buffer (10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl and 15% glycerol, pH 6.5, filter sterilized), the resulting mixture was placed on ice for 15 min, and then aliquoted 100 µl per Eppendorf tube and immediately frozen on dry ice and stored at -70°C. For the transformation itself, the competent cells were mixed with DNA and incubated on ice for 20 min, then heat-shocked for 1.5 min in a 42°C water bath and then immediately chilled on ice for 2 min prior to adding 1 ml LB broth. The resulting transformation culture was finally incubated at 37°C with moderate shaking for 1 h, before being plated on LB selective medium for incubation at 37°C overnight.

To make *W. dermatitidis* cells competent for transformations, yeast cells were cultured with vigorous shaking at 25°C in YPDB inoculated with 10^6 cells/ml. Once the culture density reached 10^8cells/ml (~1 day), the cells were collected by centrifugation, washed 2 to 3 times with ice-cold, sterile 10% glycerol and finally resuspended for maintenance or immediate use in ice-cold, autoclaved 10% glycerol with a cell density of ~$5x10^9$ cells/ml. For the transformation itself, gel purified DNA fragments were precipitated with ethanol, washed with 70% ethanol and dissolved in 5 mM Tris-Cl (pH8.0). About 40 µl of the competent cells were then mixed with 1-2 µl of the DNA (0.5-1 µg) and transferred to a prechilled 0.2 cm gap cuvett (Bio-Rad) for the electroporation in a pulser apparatus (Bio-Rad) set to 1.45 kV, 25 µF and 200 Ω. Once the pulse was applied, and the time constant was above 4 ms, 1 ml YPDB was immediately added to the cuvett to quickly

and gently resuspend the cells. The resulting transformation culture was incubated at 25°C with moderate shaking for 2-3 h, and then plated on YPDA plates containing 50 μg/ml hygromycin or SD agar plates containing 20 μg/ml chlorimuron ethyl and incubated at 25°C until colonies appeared.

2.12 Photomicroscopy

Colony morphology pictures were taken with a Canon Powershot G3 attached to an Olympus Model SZ-III dissecting microscope with a ScopeTronix Maxview Plus adapter. Light microscopy pictures were obtained with a Leica DFC camera and a Leica DMLB upright microscope (The Core Facility of Institute of Cellular and Molecular Biology, University of Texas at Austin).

Chapter 3 WdStuAp, an APSES transcription factor, is a regulator of yeast-hyphal transitions in *W. dermatitidis*

3.1 INTRODUCTION

3.11 APSES: origin of the name

Members of the fungal APSES (*N. crassa* Asm1p, *S. cerevisiae* Phd1p, *A. nidulans* StuAp, *C. albicans* Efg1p, and *S. cerevisiae* Sok2p) transcription factor family all contain a conserved APSES DNA-binding domain. In 1967, *A. nidulans* was mutated by UV irradiation, which resulted in conidation defects. One of the mutation strains was found to have a stunted phenotype, so it was named the *stua*Δ strain. In 1991, the gene for this stunted phenotype was cloned (Miller et al., 1991).

Other members of the family include the two *S. cerevisiae* APSES factors, Phd1p (pseudohyphal determinant) (Gimeno and Fink, 1994) and Sok2p (suppressor of temperature-sensitive PKA strain) (Ward et al., 1995), the *N. crassa* APSES factor Asm1p (Ascospore maturation) (Aramayo et al., 1996), and the two *C. albicans* APSES factors, Efg1p (enhancer of filamentous growth) (Stoldt et al., 1997) and Efh1p (Efg1p homolog) (Doedt et al., 2004).

3.12 Structures of APSES transcription factors

The amino acid sequences of the APSES factors are inferred from their cDNA and genomic DNA. The molecular weight of the derived protein AnStuAp is calculated to be 63.5 kD, and is composed of 590 amino acids with isoelectric point (PI) 8.48. AnStuAp is rich in serine, proline, and basic amino acids, and the majority of the basic amino acids are found in two regions: a DNA-binding domain and a C-terminal nuclear localization sequence. AnStuAp has three alpha helices around the DNA-binding domain (Miller et al., 1992). Phd1p, which is about 40.6 kD, is composed of 366 amino acids, has a PI of 8.99, and is rich in proline. *PHD1* is located at chromosome XI (Gimeno and Fink, 1994), whereas *SOK2* endodes 785 amino acids, and resides in chromosome XIII (Ward et al., 1995). Except for the APSES domain, little similarity exists between Sok2p and Phd1p. Efg1p contains 552 amino acids, with a molecular weight of 59.9 kD, and it is rich in glutamine (Stoldt et al., 1997). Efh1p, another APSES transcription factor in *C. albicans*, consists of 720 amino acids, with a molecular weight of 81.3 kD, and the *EFH1* nucleotide sequence is 45% identical to that of *EFG1*. The Efh1p amino acid sequence is 23% identical to that of Efg1p (Doedt et al., 2004). Other filamentous fungal APSES factors are generally similar to AnStuAp, differing only in peptide chain length and some modification sites. At the amino acid level, there is approximately a 50% homology between filamentous fungi APSES factors, but the homology between those of the filamentous fungal APSES factors and those of the yeast APSES factors is only at the DNA-binding domain (Gimeno and Fink, 1994; Stoldt et al., 1997; Ward et al., 1995). Because the filamentous fungal APSES factors belong to one sub-family, not only do

their amino acid sequences have higher homology than those of yeast fungi, but their flanking nucleotide sequences also have broad homology, which includes promoters and enhancers (Borneman et al., 2002).

3.13 DNA-binding sites of APSES transcription factors

The APSES domain of fungi is similar to the bHLH domain of the mammalian transcription factors MyoDp and Maxp. The tertiary structures of MyoDp and Maxp have been solved by X-ray crystallography, and the critical residues involved in DNA binding are conserved in the APSES domain (Dutton et al., 1997; Stoldt et al., 1997). AnStuAp is found to bind with MCB box, A/TCGCGT/ANA/C. It binds with the MCB boxes on the promoters of the transcription factor genes *AnBRLA* and *AnABAA* and with the MCB box on the promoter of the *AnAWH11* gene, which encodes a small, developmentally regulated heat-shock protein (Dutton et al., 1997). Efg1p is reported to bind the E box, 5'CANNTG3'. It binds with the two E boxes on the promoter of the adhesin gene *ALS8* (Leng et al., 2001). In 2004, the putative binding sites of all *S. cerevisiae* transcription factors were reported, based on statistical analysis of genome localization and conserved sequence in *Saccharomyces* species, as well as previously published evidence. TGCAGNNA and SCNGCNGG are binding sites for Sok2p and Phd1p, respectively (Harbison et al., 2004).

3.14 Expression regulation of APSES factors

AnSTUA has two transcripts, which are initiated from 1,088 bp and 1,718 bp upstream of the translation start codon, respectively, and both are terminated at 454 bp downstream of the translation stop codon. After the splicings, the two mRNAs are 3,335 bp and 3,470 bp, because there is a 497-bp intron in the 1,718-bp 5'UTR. After establishment of developmental competency, the RNA levels of both transcripts are increased, and after the induction of conidiation, the RNA level of the 3,335-bp transcript is increased five times, while the RNA level of the 3,470-bp transcript is increased two times (Miller et al., 1992). The microORFs on the 5'UTR of *AnSTUA* are reported to have effects on the regulation of the expression of *AnSTUA* (Wu and Miller, 1997). In the manner of *PHD1* and *SOK2, EFG1* has no introns. Instead, it also has two transcripts of 3.2 kb and 2.1 kb, which are initiated from 1.2 kb and 73 bp upstream of the translation start codon, respectively, and which are both terminated at 412 bp downstream of the translation stop codon. The 3.2-kb transcript is the major transcript, and the amount of the 2.1-kb transcript is only 5% of that of 3.2-kb transcript. During hyphal induction, the *EFG1* RNA level is decreased (Tebarth et al., 2003).

Using a series of *EFG1* 5' upstream sequence-deletion mutants to compare their transcription efficiency, the deletion constructs did not reveal any significant regulatory sequences, except that the TATA box region is required for *EFG1* activation. Through construction of a reporter system, the *EFG1* promoter was found to be autogenously repressed by Efg1p, and when the transcriptional repressor gene *SIN3* is deleted, the

transcription from *EFG1* promoter is not repressed. Chromatin immuno-precipitation (ChIP) revealed that Efg1p and Sin3p both bind with the *EFG1* promoter at the TATA box region, but an interaction between Efg1p and Sin3p was not detected, and electrophoretic mobility shift assay (EMSA) found no binding of Efg1p and Sin3p with the *EFG1* promoter. Probably, the binding of Efg1p to the *EFG1* promoter is weak or Efg1p is incapable of binding until other factors bind to the *EFG1* promoter. These results indicate that under repressing conditions, Sin3p and Efg1p bind with the *EFG1* promoter, causing *EFG1* expression to be repressed. Under inducing conditions, however, this kind of interaction is changed, and the promoter most likely binds with some transcriptional activators (Tebarth et al., 2003).

3.15 Biological effects of APSES factors

Overexpression of *PHD1* or deletion of *SOK2* in diploid *S. cerevisiae* yeast cells induces pseudohyphal growth, while deletion of *PHD1* or overexpression of *SOK2* generally has no effects on morphology (Gimeno and Fink, 1994; Ward et al., 1995). In *SOK2* deletion mutants, *PHD1* is up-regulated to activate the expression of *FLO11*, which encodes a cell wall glycosylphosphatidylinositol (GPI) protein required for pseudohyphal growth and biofilm formation (Pan and Heitman, 2000; Reynolds and Fink, 2001). Also, in *SOK2* deletion mutants, *ASH1* and *SWI5*, which also encode transcription factors, are induced. Swi5p is required for *ASH1* expression, and Ash1p activates *FLO11* expression. Swi5p additionally is involved in filamentous growth through activation of the endochitinase gene *CTS1* and the endoglucanase gene *EGT2* (Pan and Heitman, 2000).Sok2p

suppresses a protein kinase A (PKA) defect, inhibits sporulation, and physically interacts with the transcription factor Msn2p (Shenhar and Kassir, 2001; Ward et al., 1995). *EFG1* overexpression in *S. cerevisiae* also induces pseudohyphal growth (Rottmann et al., 2003). Furthermore, *EFG1* complements the repression of the promoter of the meiotic transcription factor gene *IME1* conferred by *SOK2* deletion in *S. cerevisiae* (Shenhar and Kassir, 2001).

In liquid or on solid hyphae-inducing media, strains of dimorphic yeast *C. albicans* lacking *EFG1* are completely blocked in true hyphal formation (Lo et al., 1997; Stoldt et al., 1997). Mutants lacking *EFG1* are not only defective in hyphal growth under aerobic conditions (Giusani et al., 2002; Setiadi et al., 2006), but also in virulence (Lo et al., 1997; Stoldt et al., 1997), white-phase colony production (Sonneborn et al., 1999b), and chlamydospore formation (Sonneborn et al., 1999a). The encoded protein of *EFG1*, Efg1p, regulates cell wall dynamics (Sohn et al., 2003), metabolic genes (Doedt et al., 2004; Lan et al., 2002), and genes associated with cellular differentiation (Doedt et al., 2004; Lan et al., 2002; Lane et al., 2001; Nantel et al., 2002; Sohn et al., 2003). Efg1p is required for the expression of many hyphae-specific genes—for example, *HWP1*, *HYR1*, *ALS3*, *ALS8*, *ECE1*, *RBT1*, and *RBT4* (Braun and Johnson, 2000; Leng et al., 2001; Sharkey et al., 1999). Of these, *HWP1*, *HYR1*, *ALS3*, *ALS8*, and *RBT1* encode GPI cell wall proteins (Bailey et al., 1996; Braun et al., 2000; Hoyer et al., 1998a; Staab et al., 1999), whereas *RBT4* encodes a plant pathogenesis-related protein (Braun et al., 2000). To date, the function of Ece1p is not yet known (Birse et al., 1993). *EFG1* is also known to be necessary for the expression of yeast cell wall-specific genes—for example, *YWP1*

(Sohn et al., 2003). In addition, *EFG1* is required for the expression of the white phase-specific gene *WH11*. During yeast-to-hyphal transitions, Efg1p functions downstream of PKA, and mutation of the PKA phosphorylation consensus sequence in Efg1p—T206A—affects hyphal growth (Bockmuhl and Ernst, 2001). Under hypoxic conditions at incubation temperatures lower than 37^{o}C, deletion of *EFG1* activates filamentous growth. In this case, another set of genes is regulated; in particular, the fatty acid biosynthesis genes require Efg1p for activation (Setiadi et al., 2006).

Overexpression of *EFG1* leads to production of pseudohyphae instead of true hyphae. The Ernst laboratory used two experiments to support this conclusion (Tebarth et al., 2003). First, they made *C. albicans* grow as true hyphae under hyphal induction conditions, and then they induced *EFG1* overexpression. The result was that pseudohyphae grew from the hyphae. Second, they induced *EFG1* overexpression to grow pseudohyphae in pseudohyphal growth conditions, and then hyphal induction conditions were applied. The result was that true hyphal growth was repressed. They found that the transcript level of *EFG1* is decreased to its lowest level during the induction of the hyphal growth, and determined that Efg1p must be repressed to allow continued apical growth (Stoldt et al., 1997; Tebarth et al., 2003). This is not without precedence. Mammalian Myc protein is required for differentiation, and its expression is decreased to low level during the differentiation process (Ayer and Eisenman, 1993). Because overexpression of *EFG1* produces pseudohyphae compared with the wild type producing yeast cells, overexpression of *EFG1* or *EFH1* activates the cell wall hyphal-specific genes, *HWP1*, *ALS10*, *RBT5*, *ECE1*, and *PHR1* (Doedt et al., 2004).

Efh1p lacks the PKA phosphorylation site corresponding to T206 in Efg1p. The phenotype of deletion *EFH1* is minor, and the induction of pseudohyphal growth by *EFH1* overexpression requires the presence of *EFG1*, suggesting that *EFH1* overexpression may activate *EFG1*, which in turn activates the pseudohyphal growth. Transcriptosome profiling shows that the genes affected by *EFH1* are fewer than and have some overlapping with those affected by *EFG1*. Reporter assays show that Efh1p serves as an activator, while Efg1p functions as a repressor. Efh1p forms a homodimer through the amino acid region before the APSES domain, but Efg1p neither forms a homodimer nor interacts with Efh1p (Doedt et al., 2004).

In contrast to the findings with the ascomycotous yeast species, the APSES transcription factor, AnStuAp, of the obligate filamentous and conidiogenous ascomycotous fungus *A. nidulans,* is best known for its involvement in conidiation. Mutant *anstua*Δ strains lack phialides and metulae (Miller et al., 1991), but still produce conidia. Although the *STUA* RNA level is induced in developmental competency, StuAp plays no role in the establishment of the developmental competency. The role of AnStuAp as an APSES transcription factor in *A. nidulans* also relates to its effects on two other conidial developmental transcription factors, AnBrlAp and AnAbaAp. AnStuAp spatially and temporally regulates the expression of AnBrlAp and AnAbaAp (Dutton et al., 1997; Miller et al., 1992). In a similar manner, deletion of *PmSTUA* in the dimorphic fungus *P. marneffei* also affects conidiation but does not influence fission of its yeast-phase cells or their transition to hyphae (Borneman et al., 2002). Although PmStuAp complements the

asexual developmental defect of the *anstua*Δ mutant, it does not complement its sexual developmental defect, suggesting that the mechanisms by which the two types of sporulation are regulated involve different pathways in different species.(Borneman et al., 2002). By comparison, deletion of *FoSTUA* in *F. oxysporum* represses the development of macroconidia and activates the growth of chlamydospores (Ohara and Tsuge, 2004). Deletion of *AfSTUA* in *A. fumigatus* results in abnormal conidiophores and conidia (Sheppard et al., 2005), and microarray analysis in *A. fumigatus* shows that AfStuAp activates numerous other genes involved in development and virulence, including secondary metabolite biosynthetic genes in the period preceding conidiogenesis (Sheppard et al., 2005).

Because APSES transcription factors are possible virulence regulators in fungi, and because the mechanisms of APSES transcription factors are far from fully understood and much different in different species, I cloned the *STUA* homolog, *WdSTUA* from *W. dermatitidis,* and then investigated the roles played by its encoded product WdStuAp on yeast mold morphotype switching in *W. dermatitidis.*

3.2 MATERIALS AND METHODS

3.21 Strains, culture conditions and microscopy

The *W. dermatitidis* and *S. cerevisiae* strains used in this research are listed in Table 3.1. Routine culture of *W. dermatitidis* was on YPD agar (YPDA) and in YPD broth (YPDB)

as described previously (Liu et al., 2004). For the detection of the filamentous growth, *W. dermatitidis* was grown on potato dextrose agar (PDA; Difco Scientific, Detriot, Mich.), potato dextrose broth (PDB; Difco Scientific, Detriot, Mich.), corn meal agar (CMA; Difco Scientific), corn meal dextrose agar (CMDA; Difco Scientific), Sabouraud dextrose agar (SDA; Difco Scientific), synthetic low-ammonium dextrose agar [SLADA; (Gimeno and Fink, 1994)], yeast peptone maltose agar [YPMA; (Ye et al., 1999; Ye and Szaniszlo, 2000)] and yeast peptone sucrose agar [YPSA; (Brown et al., 1999)]. For the induction of sclerotic forms, *W. dermatitidis* was grown in pH 2.5 modified Czapek Dox broth [MCDB; 0.1% yeast extract, 3.5% Czapek Dox, pH adjusted with HCl; (Wang and Szaniszlo, 2000)]. For slide cultures**,** a thin square of PDA agar was placed on a slide and then inoculated on each side before being overlaid with a cover slip and incubated over water in a closed Petri dish. Colony morphology pictures were taken with a Canon Powershot G3 attached to an Olympus Model SZ-III dissecting microscope with a ScopeTronix Maxview Plus adapter. Light microscopy pictures were obtained with a Leica DFC camera and a Leica DMLB upright microscope.

Table 3.1. Strains used in this research

Strain	Genotype	Reference or Source
W. dermatitidis 8656	Wild type	ATCC 34100
Hf1	temperature-sensitive (ts) hyphal-form mutant	Wang and Szaniszlo, 2000 Ye and Szaniszlo, 2000
wdstuaΔ1A	*wdstua::hph*	This study
wdstuaΔ1B	*wdstua::hph*	This study
wdstuaΔ2	*wdstua::sur*	This study
Hf1*wdstuaΔ1*	Hf1 *wdstua::hph*	This study
wdpks1Δ-1	*wdpks1::* pYEX303	This study
wdpks1ΔWdSTUA-1	*wdpks1::* pYEX303-*WdSTUA*	This study
wdstuaΔwdpks1Δ-1	*wdstua::sur wdpks1::* pYEX303	This study
wdstuaΔwdpks1ΔWdSTUA-1	*wdstua::sur wdpks1::* pYEX303-*WdSTUA*	This study
S. cerevisae MR12	*MAT a/α leu2::hisG/LEU2::pFLO11 ::lacZ::leu-2::hisG ura3-52/ura3-52*	Rottmann et. al., 2003
MR12-pYES2	*MAT a/α leu2::hisG/LEU2::pFLO11 ::lacZ::leu-2::hisG ura3-52/ura3-52* (pYES2)	This study
MR12-pYES2*WdSTUA*	*MAT a/α leu2::hisG/LEU2::pFLO11 ::lacZ::leu-2::hisG ura3-52/ura3-52* (pYES2-*WdSTUA)*	This study

3.22 Degenerate PCR and library screening

Degenerate primers with designs based on the conserved APSES domains of the StuAp orthologs of *A. nidulans* and *P. marneffei* and the Asm1p of *N. crassa* had the sequences that follow: WSF1, AARCCIMGIGTIACXGCXACXYTXTGG; WSR1: IGGDATCCAIACICCYTTXARRTGCATXGG (R=A+G, M=A+C, X=A+T+C+G, Y=C+T, D=A+T+G). PCR amplifications were carried out with 2 μM of the primers, 0.5 mM dNTP, 0.5 μg genomic DNA and 0.5 U/μl Taq polymerase mixed in 1.5 mM Mg^{++}, 50 mM KCl, 10 mM Tris-HCl (pH 9) buffer. The PCR reaction conditions were as follows: 5 min at 94°C for premelting; 50 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C for extension; 7 min at 72°C for completion of the extension. The resulting PCR products were then cloned into the pGEM-T easy vector (Promega, Madison, Wis.) and sequenced. After sequencing showed that plasmid pT-*WdSTUA*386 contained an APSES sequence of 386 bp, the full gene, which I named *WdSTUA*, was cloned from a previously constructed *W. dermatitidis* cosmid genomic DNA library (Feng et al., 2001) by hybridizion using the 386-bp *WdSTUA* PCR product as a probe. The DECA prime II DNA labeling kit (Ambion, Inc., Austin, Tex.) was used to label the probe with α-^{32}P dATP (Dupont NEN, Boston, MA). To subclone the fragments containing *WdSTUA*, Southern analysis of the cosmid digested by different restriction enzymes was also carried out with the 386-bp *WdSTUA* probe. After identifying the fragments with *WdSTUA*, they were released by digestion with *Xba*I*, Hind*III*, and Pst*I and subcloned into the corresponding sites of pBSKS vector (Stratagene, La Jolla, Calif.), yielding plasmids pBSKS-WS1, pBSKS-WS2, and pBSKS-

WS3, respectively. DNA sequencing of the plasmids was performed by the Core Facility of Institute of Cellular and Molecular Biology, University of Texas at Austin, using Big Dye technology (Applied Biosystems, Foster City, Calif.). The locations of introns, first predicted in silico by alignments and by consensus splice sequences, were confirmed by the comparison of the cDNA sequence produced from RT-PCR using one-step RT-PCR kit (Qiagen, Valencia, Calif.) with the genomic DNA sequence.

3.23 Deletion of *WdSTUA* in *W. dermatitidis*

To create the *WdSTUA* deletion construct, which contained the *hph* selection marker gene (Liu et al., 2004) flanked by *WdSTUA* 5' and 3' sequences, a 0.8-kb *Pst*I-*Sal*I fragment corresponding to the 5' region of *WdSTUA* was first released from pBSKS-WS3 and then ligated into vector pBSKS to generate pBSKS-WS4. Next a 1.4-kb *Sal*I-*Sal*I *hph* gene sequence from pCB1636 (Fungal Genetics Stock Center, University of Kansas Medical Center) was inserted into the *Sal*I site of pBSKS-WS4 to obtain pBSKS-WS5. After a 0.6-kb 3' region of *WdSTUA* was amplified from pBSKS-WS3 by PCR with primers WSF5 (GCTCTCGAGTGACTCCAAGCGACGTAAG: the introduced *Xho*I restriction digestion enzyme recognition site is underlined) and WSR5 (AGTGAAGCCAGGAACACATC), it was cloned into pGEM-T easy vector (Promega) producing pT-WS559. Subsequently, a 0.6-kb 3' region of *WdSTUA* was released by *Xho*I and *Sal*I digestion of pT-WS559 and cloned into the *Xho*I site of pBSKS-WS5 to create pBSKS-WS6. Finally, pBSKS-WS6 was cut with *Bgl*I (two *Bgl*I sites on the pBSKS vector) and *Pst*I to release the WS5'-*hph*-WS3' fragment that after gel

purification was used to transform competent *W. dermatitidis* yeast cells by electroporation as described previously (Wang and Szaniszlo, 2000; Zheng and Szaniszlo, 1999). After transformants were selected on YPDA medium containing 50 μg/ml hygromycin B (Invitrogen, Carlsbad, Calif.), *wdstuA*Δ strains were identified by Southern analysis, PCR and RT-PCR. The specific primers for the PCR and RT-PCR were designed to flank the second intron of *WdSTUA* and had the following sequences: WSF6, AAGTCGAAGCGAAGGGAGTCT; WSR6: GAGCTTTGTCCCGTTGATGAA. For the RT-PCR, RNA was extracted with hot acidic phenol from cells grown in YPDB for 24 h at 37°C and treated with RQ1 RNase-Free DNase (Promega). After the RT-PCR was carried out with the One-step RT-PCR kit (Qiagen), the amplification products were analyzed by electrophoresis in a 3% agarose gel with 2-log DNA markers (New England Biolabs, Ipswich, Mass.) as references. To disrupt *WdSTUA* with a *sur* marker, a construct with the *sur* gene flanked by 5' and 3' *WdSTUA* fragments was used. After the 2.8-kb *sur* marker was obtained by *Sal*I digestion of pCB1551 (Fungal Genetics Stock Center, University of Kansas Medical Center), it was ligated with *Sal*I-digested pBSKS-WS6 to produce pBSKS-WS7. The WS5'-*sur*-WS3' fragment was then excised by *Pst*I digestion, gel-purified and finally used for transformation. Putative *wdstua: sur* deletion strains were selected on SD agar (0.17% yeast nitrogen base, 0.5% $(NH_4)_2SO_4$, 2% dextrose, 2% agar) supplemented with 20 μg/ml chlorimuron-ethyl (sulfonylurea; Fisher Scientific, Pittsburgh, Pa.). PCR analysis using specific primers WSF6 and WSR6 was performed to identify *wdstua: sur* deletion strains by the absence of a *WdSTUA* 125-bp amplification product.

3.24 Northern analysis

Log-phase, 25°C-cultured, *W. dermatitidis* cells were inoculated into YPDB or PDB at 10^6 cells/ml. Cells were shaken at 25°C or 37°C. After 24 h or 48 h, cells were collected by centrifugation and RNA was isolated with the Qiagen RNeasy kit. The concentration of RNA was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Del.) and its integrity was detected by running a 1% agarose gel containing 2 M formaldehyde. A 0.24-9.5 kb RNA ladder (Invitrogen) was loaded as a size marker. Probes were labeled as described above. For the *WdSTUA* probe, the 386-bp *WdSTUA* fragment from pT-*WdSTUA*386 plasmid was used. For *WdCHS5* probe, a fragment amplified by PCR using primers pATG and pIntron1 was applyed (Liu et al., 2004). *WdCHS1, WdCHS2, WdCHS3* and *WdCHS4* fragments for probes were amplified by PCR with their specific primers (Wang et al., 2002). After Northern hybridization, radioactive signals were detected by exposure to a phosphoimager screen which was then scanned with a Molecular Imager FX Pro Plus multiimager system (Bio-Rad, Hercules, Calif.).

3.25 Expression of *WdSTUA* in *S. cerevisiae*

For expression of *WdSTUA* in *S. cerevisiae*, the *WdSTUA* cDNA coding sequence was placed under the control of the *GAL1* promoter in vector pYES2 (Invitrogen). The *WdSTUA* cDNA was amplified by RT-PCR (one-step RT-PCR kit, Qiagen) with primers WSF8, GAAGATCTA*ATG*AACCAAACTCAATCGTATATG, and WSR9,

GAAG*ATCT*AGTGAGGCGTCGGAGGAGCTTG (the start and stop codons are indicated by italic letters and the introduced *Bgl*II restriction digestion enzyme recognition sites are underlined). The amplified product was then cloned into the pGEM-T easy vector which, after sequence confirmation, produced plasmid pT-*WdSTUA*N containing the N terminal 1-kb *WdSTUA* cDNA. Once the 1-kb *WdSTUA*N was obtained from pT-*WdSTUA*N by *Bgl*II digestion, it was ligated to pYES2 *BamH*I site with the correct orientation to produce pYES2-*WdSTUA*N. A 1-kb *Bam*HI-*Sma*I sequence from pBSKS-WS1 was similarly ligated into the *Bam*HI-*Spe*I site (with the *Spe*I site first filled in by Klenow enzyme) of pT-*WdSTUA*N to create pT-*WdSTUA*. The *Bgl*II-*Not*I *WdSTUA* fragment from pT-*WdSTUA* was then inserted into the *Bam*HI-*Not*I site of pYES2, generating expression plasmid pYES2-*WdSTUA*. Constructs pYES2, pYES2-*WdSTUA*N and pYES2-*WdSTUA* were finally transformed into *S. cerevisiae* strain MR12, which contains a *FLO11*-promoter::*lacZ* reporter construct (Rottmann et al., 2003) kindly provided by Dr. S. Rupp (Fraunhofer IGB, Germany). To induce the *GAL1* promoter, a synthetic low-ammonium raffinose agar medium (SLARA; 0.17% yeast nitrogen base, 50 μM $(NH_4)_2SO_4$, 2% raffinose and 0.2% galactose) was used for culture.

3.26 β- galactosidase assay

Log-phase cells cultured in SD broth medium were washed, then spread on SLARA and incubated at 30°C. After 2 da incubation, cells were harvested by centrifugation, washed with Z buffer (16.1 g/L $Na_2HPO_4{\cdot}7H_2O$, 5.5 g/L $NaH_2PO_4{\cdot}H_2O$, 0.75 g/L KCl and

0.246 g/L $MgSO_4.7H_2O$), then resuspended in Z buffer and OD_{600} was measured. Cells (100 µl) were then frozen in liquid nitrogen and thawed in 37°C three times to thoroughly break the cells. Z buffer (100 µl) was used as blank control. Z buffer (0.7 ml) containing 0.27 ml β-mercaptoethanol was added. After start timing, 0.16 ml ONPG (4 mg/ml in Z buffer) was immediately added, vortexed and incubated at room temperature. When the solution became yellow, the reaction was stopped by adding 0.4 ml of 1 M Na_2CO_3 and the reaction time recorded. Once cell debris was removed by centrifugation, the OD_{420} of the sample was measured relative to that of the blank control. β- Galactosidase units = 1000 x OD_{420} / (min x 0.1 ml x OD_{600}). Two biological repeats were carried out.

3.27 Expression of *WdSTUA* in *W. dermatitidis*

The integration expression vector pYEX303 was used for the expression of *WdSTUA* as described previously (Ye et al., 1999; Ye and Szaniszlo, 2000). Briefly, pYEX303 contains the *glaA* promoter and terminator, the *hph* gene, a *WdPKS1* targeting sequence, an *E. coli* replication origin sequence, and a *Cam*-resistant marker. Transformants with putative site-specific integrations of pYEX303 in the melanin biosynthesis, nonessential, *WdPKS1* genomic locus were then identified as albino strains having hygromycin B resistance. Construction of the *WdSTUA* expression vector itself involved first cutting pT-*WdSTUA* with *Not*I, next filling in with Klenow enzyme, and then cutting with *Bgl*II to obtain the 2-kb *WdSTUA* cDNA. pYEX303 was also cut, but in this case with *Xba*I, before being filled in with Klenow, and cut with *Bgl*II, prior to being ligated into

pYEX303 to produce pYEX303-*WdSTUA*. Both pYEX303 and pYEX303-*WdSTUA* were linearized in the *WdPKS1* targeting sequence by *Nar*I prior to transformation of the *wdstua*Δ*2* (*wdstua*::*sur*) strain. *Nar*I-linearized pYEX303 was also used to transform the wild-type *W. dermatitidis* strain. PCR amplifications with the specific primers WSF6 and WSR6 were used to confirm that expression strains were derived. The morphotypes of the expression strains were characterized by growth on YPMA, YPDA, and PDA, as well as after being embedded in YPSA.

3.28 Microarray assays

I used chips kindly produced in the laboratory of Dr. Vishy Iyer (The University of Texas at Austin). These chips carried fragments representing all genes and some intergenic sequences of the *S. cerevisiae* genome. Log-phase cells of strains harboring the pYES2 or pYES2-*WdSTUA* plasmids were first cultured in SD broth medium, then collected and washed, and finally spread on SLARA and incubated for 2 da at 30°C. RNA from cells harvested from SLARA was extracted with RNeasy Mini kit (Qiagen) using a glass bead disruption protocol. RNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies), and RNA quality was determined by RNA gel electrophoresis. For reverse transcription, 15 µg RNA was with 1 µl of 5 µg/µl oligo dT in a volume of 14.5 µl was denatured at 70°C for 10 min and then chilled on ice. 6 µl of 5x buffer, 0.6 µl of 50x aadUTP/dNTPs (25 mM each dATP, dCTP, dGTP, 10 mM amino allyl-modified dUTP and 15 mM dTTP), 3 µl of 0.1 M DTT and 2 µl of SuperScript II reverse transcriptase were added to a final volume of 30 µl. The reaction

was incubated at 42°C for 2 h, followed by incubation at 95°C for 5 min and quick chill on ice. RNA was hydrolyzed by adding 13 µl of 1 M NaOH and 1 µl of 0.5 M EDTA and then incubation at 67°C for 15 min. 50 µl of 1 M HEPES (pH 7.5) was added to neutralize. The cDNA was purified with MinElute PCR purification kit (Qiagen) and eluted in 0.1 M $NaHCO_3$ (pH 9.0). The cDNA of the expression strain was labeled with fluorescent dye (Amersham) Cy5 (red, channel 2, 635 nm) and cDNA of a vector alone strain was labeled with Cy3 (green, channel 1, 532 nm) in dark at room temperature for 1 h, followed by purification with MinElute PCR purification kit (Qiagen). 1 µl of 5 µg/µl tRNA, 1 µl of 10 µg/µl poly A, 7 µl of 20x SSC and 1 µl of 10% SDS were mixed with two probes to a final volume of 40 µl and incubated at 100°C for 2 min and then cooled to room temperature. The mixture was added to the chip under a coverslip. The chip was placed in a hybridization chamber. 3x SSC was added to the reservoir of the chamber for hymidization. The chamber was sealed and emerged in 65°C water bath overnight. The chip was washed at room temperature in 0.5x SSC and 0.01% SDS for 2-5 min, and then in 0.06x SSC for 2-5 min. Liquid was spun off the chip. The chip was then scanned with an Axon GenePix 4000B scanner (Axon, Union City, Calif.) and the resulting files loaded to the Longhorn Microarray Database (LAD) for normalization (Killion et al., 2003). Three biological repeats were carried out. Undetected spots on the array were flagged and excluded from the analysis. Spots with sum of the mean intensities of both channels (532 and 635) of more than 150 were accepted. Only genes with regulation of more than 1.4 fold and greater than 80% were subjected to further analysis. The data is clustered by Cluster program and the clustering result is displayed by TreeView program (Eisen et al., 1998).

3.29 Nucleotide sequence accession number

The sequence of *WdSTUA* was submitted to the GenBank database. The accession number is AY445507.

3.3 RESULTS

3.31 Isolation and sequence characterization of *WdSTUA*

Using degenerate primers with designs based on conserved sequences in the DNA-binding domains of APSES factors of *A. nidulans* (AnStuAp), *P. marneffei* (PmStuAp) and *N. crassa* (Asm1p), I amplified by PCR a 386-bp gene fragment encoding a similar domain from *W. dermatitidis* DNA. This PCR product was then used as a probe to determine by Southern analysis that the fragment amplified was from a gene present as a single copy in the *W. dermatitidis* genome (Figure 3.1) and to screen a cosmid library to isolate the gene. Sequence analysis of 6,800 bp of one cosmid insert showed it contained the entire open reading frame (ORF) from bp 3,080 to 5,124 (Figure 3.2, 3.3). In the manner of *AnSTUA* and *PmSTUA*, the upstream of *WdSTUA* had micro ORFs; Figure 3.3 displayed two micro ORFs, encoding 74 aas and 43 aas, repectively. Comparison of the cDNA sequence amplified by RT-PCR with the corresponding genomic sequence indicated the presence of the predicted three introns of 52 , 55 and 54

bp (3,344-3,395 bp, 3,621-3,675 bp and 3,808-3,861 bp). Analysis of the ORF showed that it encoded a putative protein of 627 amino acids, with an APSES DNA-binding domain located from residues 157 to 234 (Figure 3.2, 3.3). The analysis also showed the predicted protein shared significant identity along its whole sequence with the APSES transcription factors AnStuAp (50%), AfStuAp (51%), PmStuAp (48%), and NcAsm1p (48%) from the conidiogenous euascomycoteous filamentous fungi *A. nidulans*, *A. fumigatus*, *P. marneffei*, and *N. crassa* respectively (Table 3.2, Figure 3.4A). Hence, the cloned gene from *W. dermatitidis* was named *WdSTUA* and its encoded protein WdStuAp. A phylogram derived with these same protein sequences showed that WdStuAp is in the same branch as NcAsm1p (Figure 3.4B). Few similarities outside the APSES domains were noted between WdStuAp and Phd1p (residues 123-238 in WdStuAp, 63% identity) or Sok2p (residues 132-243 in WdStuAp, 68% identity) of the hemiascomycetous yeast *S. cerevisiae*, although a number of additional identities were found in Efg1p (residues 22-422 in WdStuAp, 35% identity) of its dimorphic relative *C. albicans* (Table 3.2). In addition, WdStuAp was predicted to have a very proline-rich region (residues 84-134) containing 31% proline (Figure 3.4A), a predicted nuclear localization signal KRRK (residues 598-601), a putative PKA phosphorylation site (residue threonine 137), that corresponds to the Efg1p PKA phosphorylation site (residue threonine 206) (Bockmuhl and Ernst, 2001), several PKC phosphorylation sites, several casein kinase II phosphorylation sites, a tyrosine-phosphorylation site (residues 221-227), and three helices (residues 136-263). Moreover, WdStuAp was also predicted to be rich overall in proline (11.5%), serine (10.5%), threonine (7.2%) and glutamine (7.2%), have

a calculated molecular weight (MW) of 66.9 kDa, and possess an isoelectric point (PI) at 8.47.

3.32 *WdSTUA* is not an essential gene

To study its functions, the entire coding region of *WdSTUA,* except for a 100-bp sequence upstream of the stop codon, was deleted from the *W. dermatitidis* genome by a one-step gene replacement strategy (Figure 3.5A). About 10% of the resulting hygromycin B-resistant strains had an *hph* insertion at the *WdSTUA* locus. Southern analysis performed with genomic DNA digested with *Pst*I and *Sal*I and a 3.7-kb *WdSTUA Pst*I-*Pst*I probe showed that in contrast to the wild type that had a 2.4-kb *Sal*I-*Pst*I hybridization band for *WdSTUA*, the genomic DNA of the deletion strains (*wdstua*Δ) was devoid of this band (Figure 3.5B). In addition, whereas the wild-type strain and the *wdstua*Δ mutants both had 0.8-kb 5' region *Pst*I-*Sal*I bands, the wild type possessed an additional 0.5-kb *Sal*I-*Sal*I band, while the *wdstua*Δ strains contained a distinct 0.6-kb 3' region *Sal*I-*Pst*I band due to the introduction of a *Sal*I site between *hph* and the 3' region. When the Southern analysis was performed with genomic DNA digested with *Pst*I alone and hybridized with the same 3.7-kb *WdSTUA Pst*I-*Pst*I probe, the wild type produced a 3.7-kb *Pst*I-*Pst*I hybridization band for *WdSTUA*, while the deletion strains generated a 2.8-kb *Pst*I-*Pst*I band due to the replacement of the 2.3 kb *WdSTUA* gene with the 1.4 kb *hph* gene (data not shown). Additional confirmation of the *WdSTUA* deletion was obtained by PCR amplifications with specific primers that bound outside the second intron, which produced a 123-bp band with genomic DNA of the wild-type strain,

whereas no band was produced with the DNA of *wdstua*Δ strains (Figure 3.5C). Finally, when the *WdSTUA* deletion was investigated by RT-PCR, the amplifications showed that a 68-bp cDNA band was produced from *WdSTUA* RNA, whereas RNA from the mutants did not produce that band, thereby verifying that *WdSTUA* RNA was missing in the *wdstua*Δ mutants (Figure 3.5D). By these methods I identified five *wdstua*::*hph* strains. These were viable indicating that *WdSTUA* is not an essential gene in *W. dermatitidis*. Because a variety of preliminary studies revealed that the phenotypes of all the *wdstua*Δ mutants were identical, most of the subsequent detailed comparisons with the wild type described below were carried out with the *wdstua*Δ*1A* strain.

Northern analysis of RNA from wild-type *W. dermatitidis* cells cultured in YPDB medium for 24 h at both 25°C and 37°C showed that *WdSTUA* was expressed at similar levels under both conditions (Figure 3.6A). *WdCHS5,* which was previously found to be the only single chitin synthase gene of *W. dermatitidis* critical for virulence in mouse models and necessary for growth at 37°C (Liu et al., 2004), and therefore had been suggested to be regulated by a number of stress mechanisms, including cells shifted from 25°C to 37°C, which results in higher transcript levels (Liu et al., 2004), was also found was not to be influenced by the *WdSTUA* deletion (Figure 3.6B). Also, the levels of *WdCHS1, WdCHS2, WdCHS3, and WdCHS4* were not increased in the *WdSTUA* deletion strain by the shift of cells from 25°C to 37°C (data not shown).

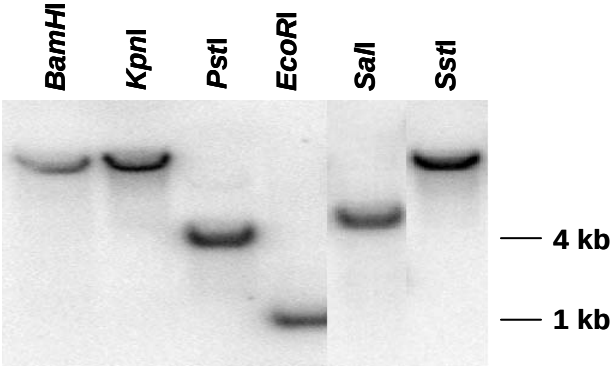

FIGURE 3.1. SOUTHERN ANALYSIS OF GENOMIC DNA OF *W. DERMATITIDIS* WILD TYPE HYBRIDIZED WITH THE *WDSTUA* PROBE. The probe is the 386-bp *WdSTUA* PCR product. Genomic DNA was cut with various enzymes.

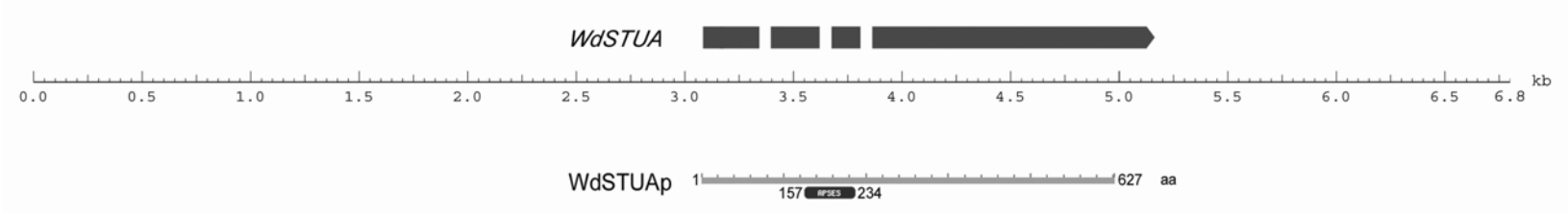

FIGURE 3.2. SCHEMATIC REPRESENTATION OF *WDSTUA* AND WDSTUAP. Diagram illustrating the cloned *WdSTUA* locus (modified from the GENSCAN program; genes.mit.edu/GENSCAN.html). The three introns that interrupt the four exons of *WdSTUA* are indicated by the open areas in the arrow.

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GAAAGCCCACGGACTGAGTTACCTTGAGAGGAGAAAGCGCTACCAGGGACCTCTTTCTGG 60
TGACATGACCACGGAACAACATGTCACACCTTGGTAGATTGGAGTTGCAATAATAATGCG 120
AGGGATACTGCGAAGGAAGGTAATCGTGAGCCGGAGGAACGCCTGAGCAAACAGGGCCTT 180
TCAACACCGTGGACCTTTGTCAGGGCTCAGGGCTTCCCTAGTCTCCTAGGCTTGTTGGAT 240
GGTGGGCGAAGGGAGGAAGCGCGGCCTGTGGAAGGATTGGGGCCTGTGGAGGCTGATCGT 300
GATAGAGGGTGAATGAGTCTCCGAGCGGCCTAACTGATAGAGCGGTACTGAGCGGTACGG 360
GGGTTTTGATGGATGTGGTGTTCTTCGTTTGTCTGGCCCAGTTGGCTTGTTCTTTGTGGC 420
TTTTGCTCCTACTGTAGAACCTAACCCAATGGCCGCCATGCCAATGGTGGGCCGATGCTG 480
ACTGGGATTTTCGAAGGCTGGGGGAAGTGGATTTGCGTCGGTAAACTCGAGGTCTGTGTG 540
TCCGATTGTCATCNGGCGCGGTTGACGTCCTTGTGTCCGTTCTCCTCTCTGCTTTGGACG 600
CGTTGCGCTATACCCCCAAAGTCATTATTTGCAGGCTGAATTCGCCAAAGCGAAGAGAAA 660
ACGGCAGGGATTGGGGACAGGACAGCAAGGTGGAAGTGCCCTGCCGACGCCACAGTATGT 720
TCCCCGGTTGCGTCCACGGCTTACAGCACCCCTTCAACAACAGTACAATTGTACCGCTGC 780
AGTAAGAATCGTAATAGAGGCCCTCAAGACTGAGCGGATGAACCTTTCCCGTAACGGTGA 840
AAGTCACGCAGAACATGCAGCCCTAAGTAGAGAACCCCCGCCGTCCGCCGACCAAGGCCG 900
AATTGTCACGATTAGGACCCATGCCGGAGTTGAAGGTTCCGAACTATCACACTGTTGGCT 960
CCTTCTATTATTCCATTTTTCCGTTGGTGAATATCGTTGGTGTGAGACAGTGCCCGCAGA 1020
GAAGATACGAAGGGTTCGCTAGCCTCGTAGCTGGAGATTGTAAGAGTTTGATGGGAACAT 1080
TATTGTTCAGGTGTATCTACTGGCCGATCCGCCTCTGGAATCGACAATCATCATGCCGGT 1140
GGCAGGGAGCTGGAGGTCCGTTGGTTCAACCAGCATCCCTGCAAGAGCAAGGGTAGCGAG 1200
GTACTGTGTATTTTATTGATGAGCAGGTTCAACAGTAGCCGTGCAGTGAGGCGTTCTGGT 1260
GCGTGGTGTTGTGCGAAAAGGCACCCAGCCCTCCCCGGCTGTGATTGGCAGTCAGTATCC 1320
GGCCGCCGTGGGTCTTTGTTGGGCCTCTCGTGGATCTCTGACTCGCAACTTGCGGCATGC 1380
TGGGATCGTAGGAGCGTGGGCTGGTGGACAGAGGCGCGCCATGGGCGGCCGGCAGTGGTC 1440
GGTGGAGCATGATATGGAAGGACAAGTCTAGGAAGAGCGAGCCCGGGTTCCAATGAGCGA 1500
GGGGCGGTGGGCAGTGGGCGGTGGGTGGCCAGCAGATGGTCCAGCAATGCCAGCATAGCG 1560
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GAAGAAGCAGCGGTTGGGTGGTTAGCTGTTGGCTAGTAGTTGTAGCTAGTTGTAGTAATC 1740
GTGGTGTTGGTGGTGTCGGCGGTGGCCGTGGGTGGTTGTGGGCGTGGTCCTGGTTGTGGA 1800
CGTGGCTGCGGTGCAGGCCAGTGGTTAGCATCCCAGTGGTTAGCATCCAGGTGGTTAGCG 1860
GCAGTGGTCATGGTGGACTGCGGTGCCAGTGCCAATGCCTGCGGTGGATGAATCGGTGGA 1920
TGAATCCCTGCAGTGCCAGTGAGTGGTCCTCTCTGCGCTCTGCTGGCTGCTGCTGGCCCG 1980
CTGGCTTTTTTCTGCTGCACCACTTCCGCTTCTGCCTTTTTGCTCTAATGCAGCGACCTG 2040
CACACCCTCCCTCCTCCCGCTCGGCTAGGTTGCGCACCGTCGTCCTTCTTGCATTCCTGC 2100
ATCCTGCTTCAAACTGAACCTCACTACTTGCCTCTGGCCTCCAGATCTCCATTGACACTT 2160
TTTGTGCATCCATAGTCGCCTTGTCCTGTGGTCACCAGTCAGTCATGGCCTAATAGCGCG 2220
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TACGGGTCTCACAGAATGGAATGCGTGATGTTCCTGCATATCAAGCTGCCTCTGTCTGGA 2460
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TTCCTGTGTGGCTTCTTCCCCGCGGCACGCTCAATAGAGGTTGACCATTGAGGGCCGTCG 2580
CCAGTTCTGACAAAAGGGCGACTCTTGGTGCCTCTACGAGTCTGTACAGCTGTACAGTCT 2640
GCCTGCGACGCTGTTTCTTCATTGAAAGGCGGAATACCTGGCTCGACGTCTGACTGGCTT 2700
GTAAAAGGCCGGACGGAACGATAAGGCTACGGTTGACAGGCATCAGAGTTTGATCGTTGT 2760
ATTTTAACAGTCGACGCGGCTGTTTCCCGAATTCAAACTCTGCGTTGTGGAAGACCGATC 2820
ACCGGCGGTGGAACGCCCACTTGATCTCATCTGTTCGACTACCGTCCGCACTTCGATTAA 2880
GAGGAGGGATAAATTTGGTGTCGATCCGTATACGTCTGCCATTGGTCTCATCAGGCTTGG 2940
GTCTCGTCCTGGAGTAAGAGATATAATTGAAGCGCACCGTCAGGCTCTCAGAGGGTGGCC 3000
CTTGTGTCCTTTCTTTGTATCAAGCCTTGCGAAGAAGAAAAGTGGTTAAGCTGTCCGCTT 3060
GCCACAGGCCCAAACCAAAATGAACCAAACTCAATCGTATATGGACATGCATTCTGCCTC 3120
                  M  N  Q  T  Q  S  Y  M  D  M  H  S  A  S  14
GCACATGTCTTCCGGGCCGTCATATTCACATCCCGCAGGCACTGCTCCTTTGGGTCACTA 3180
 H  M  S  S  G  P  S  Y  S  H  P  A  G  T  A  P  L  G  H  Y  34
CCAGCAATATCAGCAGCCTCCGATTATTCCCCCTGCGTCGACGCATTACGGCCCTCCACA 3240
 Q  Q  Y  Q  Q  P  P  I  I  P  P  A  S  T  H  Y  G  P  P  Q  54
GGCGTATGGCAATTATGGCTATGCCAATGGAGTGACATCGCCGCAGTCTGCTGCTGGCCA 3300
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 A  Y  G  N  Y  G  Y  A  N  G  V  T  S  P  Q  S  A  A  G  H  74
TGTATCGCAGCAGGTGCAGTCGCAGATGCCGTCCCTTCCAGGTGAGCACGATCCATCATC 3360
 V  S  Q  Q  V  Q  S  Q  M  P  S  L  P  A                    88
GACCATTGTACTCATTTGCTGACTATCCCACAGCCATGATGCCAGGGCCTAGCCCTCAAC 3420
                                    M  M  P  G  P  S  P  Q  Q  97
AGACTTACGTGCCACAAACCTCTAGTTTGCCTGCTCCTGCTCAGGGATACCCCCCGGCCC 3480
  T  Y  V  P  Q  T  S  S  L  P  A  P  A  Q  G  Y  P  P  A  P  117
CGCCAATTCCATTCGACACGACTGGCCAGATTGCTCCACCTGGAATGAAGCCTAGGGTGA 3540
  P  I  P  F  D  T  T  G  Q  I  A  P  P  G  M  K  P  R  V  T  137
CAGCGACACTGTGGGAGGATGAAGGAAGTCTTTGCTTTCAAGTCGAAGCGAAGGGAGTCT 3600
  A  T  L  W  E  D  E  G  S  L  C  F  Q  V  E  A  K  G  V  C  157
GCGTGGCTCGTCGTGAAGGTAGGCTCCATACTCGACAATCTTTGACCTGGACAGATATTT 3660
  V  A  R  R  E  D                                            163
ACGTGGGCTCTAGATAACCACTTCATCAACGGGACAAAGCTCCTAAATGTTGCTGGCATG 3720
                N  H  F  I  N  G  T  K  L  L  N  V  A  G  M   178
ACTCGTGGTCGCCGTGATGGCATACTGAAATCAGAGAAGACGCGACACGTCGTCAAGATC 3780
T  R  G  R  R  D  G  I  L  K  S  E  K  T  R  H  V  V  K  I   198
GGTCCAATGCATCTCAAAGGTGTTTGGTATGTCGCCAACTTGCTCCATGCTTCAAGGTCG 3840
G  P  M  H  L  K  G  V  W                                   207
CATTGCTGATTGCGGTACAGGATCCCTTTTGAGCGTGCGCTGGAATTCGCAAATAAGGAG 3900
                   I  P  F  E  R  A  L  E  F  A  N  K  E   220
AAGATTACCGATCTTCTATATCCTTTGTTCGTGCACAACATTGGTGGCCTGTTGTATAAC 3960
K  I  T  D  L  L  Y  P  L  F  V  H  N  I  G  G  L  L  Y  N  240
CCGGAGAACGCGCCTAGAACAAATGCAGTTGTGCAAGCAACCGAGCGCCGTCGTATGGAC 4020
P  E  N  A  P  R  T  N  A  V  V  Q  A  T  E  R  R  R  M  D  260
AGCATTGATGCGCCACGGTCTTCGCAGCCTTCACAGACACCATCCCTCCCACTTCATAGT 4080
S  I  D  A  P  R  S  S  Q  P  S  Q  T  P  S  L  P  L  H  S  280
ACAATGTCTGGCAGTGGCCAAGCTCCTCCGACGCCTCACTCGGTCGCGCAACACGCCAAT 4140
T  M  S  G  S  G  Q  A  P  P  T  P  H  S  V  A  Q  H  A  N  300
ACGCAACGCCCCGGCATCGACAGGGCTCACACTTTTCCTACGCCTCCCACGAGTGCTTCC 4200
T  Q  R  P  G  I  D  R  A  H  T  F  P  T  P  P  T  S  A  S  320
AGTGTCATGGGTATGACCAACCAAGGCAGTTCATACGACTGGAACAACCAGGGCTTGGCA 4260
S  V  M  G  M  T  N  Q  G  S  S  Y  D  W  N  N  Q  G  L  A  340
AATGGGCCGGCCGGCACGCAACCTTTGTCTATCGACACCGGTGGGATCAACGCAAGGTCC 4320
N  G  P  A  G  T  Q  P  L  S  I  D  T  G  G  I  N  A  R  S  360
ATGCCGACAACGCCTGCCACCACTCCGCCAGGTACTGCCGGACAAGGAATGCATCCTTAT 4380
M  P  T  T  P  A  T  T  P  P  G  T  A  G  Q  G  M  H  P  Y  380
CAGTCGCAGTCGAGTTACGACAACTCGAAGCATTATTACAGTACCACGCCGAGTTCTCAA 4440
Q  S  Q  S  S  Y  D  N  S  K  H  Y  Y  S  T  T  P  S  S  Q  400
ACTGGTTATGCACAGCACCCCGATGGGAGTCGATACGGCCAGCACATGCAAGCCGTTGGG 4500
T  G  Y  A  Q  H  P  D  G  S  R  Y  G  Q  H  M  Q  A  V  G  420
TACGGGAAGAGTGATATGGGGCCTCCCATAGCACCGGGGAGCGGTAGCGGCGGCGGGGAC 4560
Y  G  K  S  D  M  G  P  P  I  A  P  G  S  G  S  G  G  G  D  440
GGCCACGACCACAAAACTGACACGTACCCTGGCCAAAATCATAGCTCTCAACATGAGGGC 4620
G  H  D  H  K  T  D  T  Y  P  G  Q  N  H  S  S  Q  H  E  G  460
GATCATACGGACGGCGGTTACATGAATGCAAACGCCTCCGCCTACAATGCAAACCGTGGA 4680
D  H  T  D  G  G  Y  M  N  A  N  A  S  A  Y  N  A  N  R  G  480
CATTATGCATACGGCGCACCAGGTGACCATTCTCACTTGTCTCCTGAACAAATGTCCGGT 4740
H  Y  A  Y  G  A  P  G  D  H  S  H  L  S  P  E  Q  M  S  G  500
TCTCCGACCCATCAAGCTGGATCTGGTCACGCCACGCCACGAACCATGCCTTCCGGTCAA 4800
S  P  T  H  Q  A  G  S  G  H  A  T  P  R  T  M  P  S  G  Q  520
CCACAGTGGACGCCGGATTATCATACTCCGCCGAACCGGGCACCTCCGTCGAGCAATGTT 4860
P  Q  W  T  P  D  Y  H  T  P  P  N  R  A  P  P  S  S  N  V  540
TATAACATCATGGGTCGTGCTCGAACCCCACAAGGAGGCTCTGCCGATGGGTATGGCAAT 4920
Y  N  I  M  G  R  A  R  T  P  Q  G  G  S  A  D  G  Y  G  N  560
TCGACATATTCCAGCGGAGGCCATGCGGGGGCCATGTCATCTGCCAAACGCGGACGAGAG 4980
S  T  Y  S  S  G  G  H  A  G  A  M  S  S  A  K  R  G  R  E  580
GACGACGATCAAGATCGACCAAGCAGTCGAGATTATGACGGTTATGACTCCAAGCGACGT 5040
D  D  D  Q  D  R  P  S  S  R  D  Y  D  G  Y  D  S  K  R  R  600
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AAGATGGGACGACAAGAGACATTTGGAATGCCTCTCAATCCACCTCCACATATGCAAGCG 5100
K  M  G  R  Q  E  T  F  G  M  P  L  N  P  P  P  H  M  Q  A   620
ATCAAATCCGGCGGACAGCGCTGATTGCCGTTACTTGTCTCTTTTGGGACAGGGTCGGTT 5160
I  K  S  G  G  Q  R  *                                        627
CGCGGACGCGTATGCCCGGGCATGACAACTGGATAAATGGCTTGCAGAACGAATGATAAG 5220
CTCTGATTCCGAGATGACGCGAAGTCACGACAGATCCGAAAAGCGATTACGATATTACGA 5280
ACGGCTACAGCCAAAAAGAGATCAAAGACTAACGAACTCAATAAGCATGCGTTTATTGCT 5340
GCCTCGAAATTTTAACGACTTACGACACGATTTTCTTTTATTGTCTTGCAGATGGCGAGA 5400
TGTGACGACTGCCTTTCCGCTCATGGCCTTTCTTCGACTGGCCAGTGGTTGGTTTAGATG 5460
TGCTGCTTGCTGGTTTGTCTTTTTCTGTGTATGAGGGAATGATATACGTCTGGATGGATC 5520
ATGATTGAGACTTGAAGGATACGAATGCCACGGCGCTTTTCTAGATGTGTTCCTGGCTTC 5580
ACTGCAAGGCGGGCCGATCGAGTTCTGCAGCGACACTGCGCTCAAGCTGGACTGCCTTGA 5640
TCCCTTTGCTCCGAACCTCGCCTCCTGTAGTGCGTTGTGTTCGGAATGAAGGGGCTGGCT 5700
TGCAGGGCCAGTACATTAGCCAACAAGGAGCCGTCTAGAGAAAGAGGTGGCTCTCGCATC 5760
CTCCTGAGGCAATCTCGCGATGACATGTCAAATACAATAAAAGTCTATTTGTAATCATTC 5820
ATGAACCTTTTCGAGTTCGAGTTCTTCGACCTCGATTTCGGGCTGTCGGTGTTCAGATCT 5880
GCCCTCAGCTTGATCAAAGGAAAGATGTTCTGATCCTTGTAGGCTACCGACGGCTTTTCG 5940
AGACAAAACGACACGATGGAACATGAACAGCGGAGCCATAAAGGTAGGTCCCCATGTCCC 6000
AATGTCATGTCCTATTCGGTCACATCCATGCCGTGCAGTTGCAAGCAGCAGCATCGAATC 6060
ACGCGGTAAATATCGTGTGTACGTTGTACCTGTATGTGCTGTCCGAATCAGTAGACAGTC 6120
CAGTGTGTTGGTTGTTGGTGTTGCCGAGGACGTCTTGGCCAACTTCTCAACCGTACCTGT 6180
AAGTGATGCTAAGGAGGGTATAGCACTTATCTGACCTAGGTGCTCAGGCAATGATTGACG 6240
GGCGGCCTTCCTGATAGCAACGGACACGTCTAGAGTAATACCACGGCGTGCTTCTAGGGG 6300
AGGAGGCAGGGACACTAGTAGGGGAGGCGACGGGTGAGAGGACGAGGTATAGCGGAAGTA 6360
GAAATAGGGAGGGCTCCCGACATATAGGGGGGATAGACCTACATGGACCCGGAGAGGGAC 6420
TGGATTAGGTATAAGAAATAGTACTACTACCTACCAGTCCCTGGAGACGCTACTGGCTAT 6480
TATGGCATGCGCAACTGCAATGCTTCTACTGTTATTGCAGATTGAAAGCAAAACTCAACC 6540
ATTGCACCAATCCTCTGCAAAATAACGACCACGATGACGACGACGATGGTGGTGGTGCTG 6600
GTGGTGACGGCGATAATCTCACGTCCGAAACTCATGTTCGGTGAGGATTTGCCGCACCGA 6660
CACAGGCCTGGCGACCTCAGTCAAAAGTCCCAGCTGCTGGATCGCATCGCCGTGGCATCG 6720
GATCACATCTGGATCGATTGAAGAGGTTTGTACAACGACCTTGCCATGGCAATGCCGGTG 6780
AGCGACCACATTCCAAGCTT                                          6800
```

FIGURE 3.3. *WDSTUA* NUCLEOTIDE SEQUENCE AND ITS DERIVED AMINO ACID SEQUENCE. The amino acids for the design of the degenerate primers are shown in bold, the sequences of introns are indicated by italic letters, the putative micro ORFs are shown by dash lines, and the APSES domain (based on Pfam database) is underlined by a thick line and its proline-rich profile (predicted from Pfam and PROSITE) is underlined by a thin line.

A

```
AnStuAp         MASMNQPQPYMDVHS-HLSSGQTYASHPATAGALTHY-QYPQQPPVLQPTST-YGPASSY 57
AfStuAp         ---MNQTQPYMDVHSSHLSSAQPYASHAATAGAMAHYPQYHQQPPVLQPAST-YGPASSY 56
PmStuAp         ---MNQTQSYMDVHTSHFSSPQPYGSHGATAGGMVPYSHYQQPPPLLPPGSAGYPSTPGS 57
WdStuAp         ---MNQTQSYMDMHSASHMSSGPSYSHPAGTAPLGHYQQYQQ-PPIIPPASTHYGPPQAY 56
Asm-1p          ---MN-PNTPADVYYGQMSQG---SSMPVTTVPSHSHYASQQPPPLLQPGST-YAHQYGT 52
                   ** .:.  *::     .     *  . :     :    * **:: * *: *    .

AnStuAp         SQYPYPNSVASSQSVPPP-TTSISSQVP--AQLLPLP-VTNHPVPTHGYGNNSGT---PM 110
AfStuAp         SQYAYPSGVASSQTAPPPPSTSMSSQVP--AQLLPLP-VNSHTVTAPGYGNTTGT---PM 110
PmStuAp         YSYPYSNGVASTT---QPASNSISSQVP--AQILPLPAMTSHTVTPHGYVSGAAQ---SQ 109
WdStuAp         GNYGYANGVTSPQSAAGHVSQQVQSQMPSLPAMMPGPSPQQTYVPQTSSLPAPAQGYPPA 116
Asm-1p          PQYGYANALSSPASIPPSLPPSMNSMAG--QSVLPLPGSGSMNPAVYASGG--------- 101
                 .* *...::*.       . .:.*    :  ::* *   .   .  .

AnStuAp         QGYVYDPTGQMAPPGAKPRVTATLWEDEGSLCYQVEAKGVCVARREDNGMINGTKLLNVA 170
AfStuAp         QGFVYDTTGQLAPPGAKPRVTATLWEDEGSLCYQVEAKGVCVARREDNHMINGTKLLNVA 170
PmStuAp         QNAVHDPTGQTCPPGAKPRVTATLWEDEGSLCYQVEAKGVCVARREDNHMINGTKLLNVA 169
WdStuAp         PPIPFDTTGQIAPPGMKPRVTATLWEDEGSLCFQVEAKGVCVARREDNHFINGTKLLNVA 176
Asm-1p          ----FDTTGQVAPPGMKPRVTATLWEDEGSLCFQVEARGICVARREDNAMINGTKLLNVA 157
                    .*.*** .*** ****************:****:*:******** :**********

AnStuAp         GMTRGRRDGILKSEKVRNVVKIGPMHLKGVWIPFDRALEFANKEKITDLLYPLFVQHISN 230
AfStuAp         GMTRGRRDGILKSEKVRHVVKIGPMHLKGVWIPFERALEFANKEKITDLLYPLFVHNIGG 230
PmStuAp         GMTRGRRDGILKSEKVRHVVKIGPMHLKGVWIPYERALDFANKEKITDLLYPLFVHNIGG 229
WdStuAp         GMTRGRRDGILKSEKTRHVVKIGPMHLKGVWIPFERALEFANKEKITDLLYPLFVHNIGG 236
Asm-1p          GMTRGRRDGILKSEKVRHVVKIGPMHLKGVWIPFERALDFANKEKITELLYPLFVHNIGA 217
                ***************.*:***************::***:********:*******::*.

AnStuAp         LLYHPANQNQRNMTVPDS--RRLEGPQPVVRTPQAQQPPSLHHH-SLQTPVPSHMSQP-- 285
AfStuAp         LLYHPTNQTRTNMVVQESQQRRLEGP-PSARTPQASQPPALHHHHSMQTSIPSQMPQPPT 289
PmStuAp         LLYHPANSNRTNMVVHDSQQRRLEGS-QTARTSQGPQAPALHHHHSMNGSVPSHMPQASA 288
WdStuAp         LLYNPENAPRTNAVVQATERRRMDSI-DAPRSSQPSQTPSLPLHSTMSGSGQAPPTPHSV 295
Asm-1p          LLYHPTNQSRTSQVMAAAEQRRKDSHGQLRGPPGLPSLQQHHHHHSMLPGPPSLPSHPS- 276
                ***:* *  : . .:  :  ** :.      ..   .      * ::     :  .

AnStuAp         -----GGRPSLDRAHTFPTPPASASSLIGITSQNNSYDWN-PGMNSSVPNTQPLSIDTSL 339
AfStuAp         MSSQPGARPPLDRAHTFPTPPASASSLMGLSNQSSSYDWNNQGMNSGVPNTQPLSIDTTL 349
PmStuAp         STPQTNGRPELNRAHTFPTPPASASSLIGIPNQGSTYDWNSQNINSTVQTSQNVPIDNGL 348
WdStuAp         AQHANTQRPGIDRAHTFPTPPTSASSVMGMTNQGSSYDWNNQGLANGPAGTQPLSIDTGG 355
Asm-1p          -----MGRPALDRAHTFPTPPTSASSVMGPMGNSDGYQWSQQSMS-GTQGNSSLSLDTSL 330
                      ** ::*********:****::*  .:.. *:*.  .:      .. :.:*.

AnStuAp         -SNARSMPTTPATTPPGNNLQGMQSYQPQS-GYD-SKPYYSAAPSTHPQYAPQQPLPQQS 396
AfStuAp         -SNTRSMPTTPATTPPGNNLQGMQSYQSQS-GYDTSKPYYSTAPPSHPHYAP-----QYS 402
PmStuAp         -NSTRSMPTTPATTPPGNNLQGMPPYQNQP-AYDSSKSYYSAAPSSQAQYAS-QPLPAHS 405
WdStuAp         -INARSMPTTPATTPPGTAGQGMHPYQSQS-SYDNSKHYYSTTPSSQTGYAQ-----HPD 408
Asm-1p          GSNARSMPSTPATTPPGSTIQSMQNYPPVSQSYESSRQMYQGQSAQQAQYQSQQHYSSQP 390
                  .:****:********.  *.*  *   . .*: *:  *.  .. :. *

AnStuAp         MAQYGHSMPTSSYR--DMAPPSSQR-GSVTEIES-DVK----TERYGQG-------TVAK 441
AfStuAp         LSQYGQPMPPHSYIKNEMAPPAGRAPGGQSETETSDVKP---ADRYSQSNGHVT-AGAGE 458
PmStuAp         LT-YGQPMMK------DLGS-SGRPPLGPVEQEHDEVK----VDRYNQPNGQVTNGTEEE 453
WdStuAp         GSRYGQHMQAVGYGKSDMGPPIAPG-SGSGGGDGHDHK----TDTYPGQN-----HSSQH 458
Asm-1p          QHQERPVYSQSSYIKNDMGPPSGRPTGQSNDASDSKPPTGMIHQGQGQSDPGTHAGSEED 450
                               ::..  .         .  .      :              .

AnStuAp         TEPEQEQEYAQPD-SGYNTGRGSY-YTTNPSVGGLAHDHSQLTPDMTGSP-QQNGSGRMT 498
AfStuAp         SAPEHESEYVQHDNTGYGASRSSYTYTTNTSVGSLAGEHSQLTNDITGSP-QQNGSGRMT 517
PmStuAp         NGQQQEPEYVQDNVAGSYANRNSYTYTTNPSVSSLSGDHSQLG----GSPSHQNGSDRMT 509
WdStuAp         EGDHTDGGYMNANASAYNANRGHYAYGAPGDHSHLSPEQ------MSGSPTHQAGSGHAT 512
Asm-1p          DDANNEAEYTHDS-GGYDANRGSYNYNTQ-AVNSLPHDHG-LAPEIGGSP-HQAGSGRAT 506
                   . :  * : .  .  :.*. * * :    . *. ::      : *** :* **.: *
```

```
AnStuAp         PRTSN--TAPQWAPGYTTPP-RPAAASSLYNIVSDTRGTSG--------ANGSTSDNYSV 547
AfStuAp         PRTGGG-PPPQWASGYASP--RPTAASSLYNIVSDTRGSS----------NGAGSENYTV 564
PmStuAp         PRTAGTNPPPQWSQGYNTPP-RAVPAGSISNIVSDTRGAPN--------GDSYAPGTAYA 560
WdStuAp         PRTMPS-GQPQWTPDYHTPPNRAPPSSNVYNIMGRARTPQ----------GGSADGYGN 560
Asm-1p          PRTAAAPSSYYSAQGYHTPP-RGQPSSSLYNVMSNERTGSNGTQGNEMYAGQADMPSSLP 565
                ***         : .* :*  *  .:..: *::.  *

AnStuAp         ASNSGYSTGMNGSMGSNKRMRDDDDDRIVPPDSR-----GEFDTKRRKTLTETPVGGPVG 602
AfStuAp         ASNTAPTYSMGGSLGSGKRGREDDD--MGRPDSQ-----GDYESKRRRTN-ETTVGGPVG 616
PmStuAp         SNYSGYSSVNGSSMGSTKRMRDDDDDHLSRSDGRE----NEYETKRRKTLTEPPVGG--A 614
WdStuAp         STYS--SGGHAGAMSSAKRGREDDD--QDRPSSRDY---DGYDSKRRKMGRQETFGMP-L 612
Asm-1p          NGYSAQPSVMNGSSGGLKRGRDDDDD-GGRPTTSAPNLGPGMDMKRRKTMMDGGSLPSPT 624
                   :  .    .: .. ** *:***     .           : ***:   :

AnStuAp         GVPLGLQPMKAGGSLISARR 622
AfStuAp         GVLLGLQPMKAGGAMPRRR- 635
PmStuAp         FMQMQQQPVPAGGVMRRR-- 632
WdStuAp         NPPPHMQAIKSGGQR----- 627
Asm-1p          YTATIAQAAPSAIAAHRRR- 643
                      *.  :.
```

B

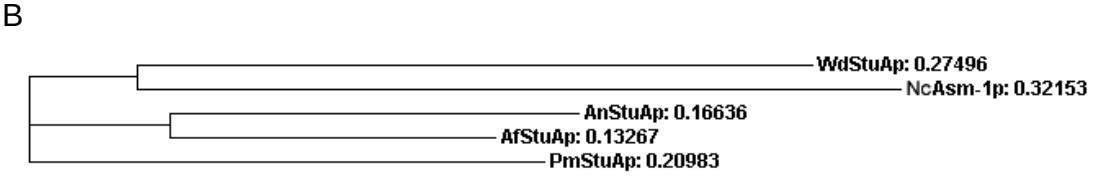


FIGURE 3.4. MULTIPLE ALIGNMENT AND PHYLOGRAM ANALYSIS OF WDSTUAP WITH ITS CLOSELY RELATED APSES PROTEINS BY CLUSTALW PROGRAM. (A) *A. fumigatus* AfStuAp accession #: EAL93087, *A. nidulans* AnStuAp accession #: AAA33325, *P. marneffei* PmStuAp accession #: AAM27919, *W. dermatitidis* WdStuAp accession #: AAS01026, *N. spora* Asm-1p accession #: AAB06995. Stars indicate the identical amino acids, two dots show the similar amino acids, and single dots present the less conserved amino acids. The APSES domain (based on Pfam database) is underlined by a thick line and its proline-rich profile (predicted from Pfam and PROSITE) is underlined by a thin line. (B) Phylogram exhibits the evolutionary relationship among these protein sequences.

Table 3.2. Homology of WdStuAp to various StuAp family members by BLASTP search

Organism	WdStuAp homologs	Identity (%)	Positive (%)	Homologous region in WdStuAp (aa*)	Score (bits)
Aspergillus fumigatus	AfStuAp	51	62	1-625	413
Penicillium marneffei	PmStuAp	48	59	1-601	365
Aspergillus nidulans	AnStuAp	50	59	4-601	337
Neurospora crassa	Asm-1p	48	57	23-554	320
Candida albicans	Efg1p	50	59	22-234	161
Saccharomyces cerevisiae	Phd1p	66	77	123-234	140
	Sok2p	70	81	132-238	134

*aa, amino acid

3.33 *WdSTUA* mediates morphological modulations in response to 37°C

The deletion of *WdSTUA* did not affect growth, melanin deposition, or cellular morphology on YPDA (YPD agar) and in YPDB (YPD broth) at 25°C. However, because 37°C is the infection temperature *W. dermatitidis* encounters when introduced into the human body, the effect of this higher temperature on growth on YPDA of the *WdSTUA* deletion mutant was also compared. The results revealed that the growth patterns of the wild type and *wdstua*Δ*1A* that developed at 37°C were similar (Figure 3.7A). However, the *wdstua*Δ*1A* strain produced colonies with surfaces that were considerably more convoluted than those of the wild type, although both types of colonies were darker and more convoluted than the colonies produced at 25°C (Figure 3.7A). In addition, the morphotypes making up the colonies of *wdstua*Δ*1A* were two times more hyphal instead of yeast as compared to those of the wild-type colonies (data not shown). Microscopic observation of the strains cultured in YPDB at 37°C additionally showed that the hyphae of *wdstua*Δ*1A* were more likely to be aggregated than were those of the wild type (Figure 3.7B).

3.34 *WdSTUA* deletion blocks filamentation and consequently conidiation on mold-inducing agar media

Because *STUA* in some filamentous fungi is involved in conidiation, we evaluated the effects of the *WdSTUA* deletion on this process in *W. dermatitidis*. Cells were streaked on PDA, a mold-inducing medium, and incubated at 25°C. The wild type and

*wdstua*Δ*1A* both increased the diameters of their initial central yeast colonies equally. However, the colonies of the wild-type formed many long branching hyphae at the edge of colonies, whereas *wdstua*Δ*1A* produced considerably fewer hyphae, which were also much shorter (Figure 3.8). On three other mold-inducing media, CMDA, CMA, and the synthetic low ammonium dextrose agar (SLADA, 50 μM $NH4^{+}$) medium, *wdstua*Δ*1A* showed similar defects in hyphal development (data not shown), whereas on another medium for molds, SDA, or when the ammonium concentration of the SLADA was increased 10 times, neither the wild type nor *wdstua*Δ*1A* formed equivalent filamentous growth (data not shown). Interestingly, when cultured at 37°C on PDA, CMA, CMDA, and SLADA, the *wdstua*Δ*1A* produced again fewer and shorter hyphae than did the wild type, but considerably more than at 25°C (Figure 3.8), suggesting 37°C suppressed the inhibition of filamentation caused by the *WdSTUA* deletion. Because of the similar phenotypes produced on PDA, CMA, CMDA, and SLADA, only PDA subsequently was used for the characterization of the filamentous growth.

To observe conidiogenesis microscopically, PDA slide cultures were prepared in a way that aerial hyphae and conidiophores, if produced, would grow horizontally out of agar into the air between a slide and a cover glass. At 25°C, the wild type, as expected, produced in the air mostly long, thin true hyphae. Along these hyphae, numerous yeast-like cells were observed at septation regions. After two weeks of incubation, the wild type was also observed to produce clusters of terminal conidia from relatively undifferentiated conidiophores in the manner characteristic of *W. dermatitidis* (Figure 3.9). In contrast, *wdstua*Δ*1A* was strongly repressed in aerial hyphal growth and

conidiation. When the infrequent filamentous growth of the *wdstua*Δ*1A* mutant was observed critically, its hyphae were shorter and largely covered with yeast cells (Figure 3.9). Thus, without WdStuAp, aerial hyphal development was dramatically reduced in *W. dermatitidis*, which consequently inhibited conidiation.

3.35 The *wdstua*Δ*1A* mutant is defective in invasive growth

Hyphal growth *in vitro* and in vivo often includes both aerial hyphae and invasive hyphae. Because the *WdSTUA* deletion produced hyphal growth defects, and invasive growth is sometimes important for fungal penetration of human tissues, the wild type and *wdstua*Δ*1A* were examined *in vitro* for their ability to produce invasive growth (growth that penetrates an agar medium). After eight days, the yeast colonies of the mutant strain produced at 25°C on the surface of PDA plate medium containing 2% agar had formed very few hyphae, whereas the wild type developed luxurious filamentous growth around and over the surface of the central yeast colonies (Figure 3.10A). After the surface growth of the colonies was washed away, comparisons revealed that the wild-type strain had invaded the agar medium considerably more than had the mutant (Figure 3.10B, C). Micoscopic examination showed that the invasive growth of the wild type consisted of both yeast and long true hyphae, whereas that of *wdstua*Δ*1A* consisted mostly of yeast (Figure 3.10D).

3.36 Deletion of *WdSTUA* in the Hf1 strain

The Hf1 strain of *W. dermatitidis* is a temperature-sensitive (ts), hyphal-form mutant that previously was reported to produce yeast cells in the manner of wild type in rich broth at 25°C and to generate many more filaments than wild type under the same conditions at 37°C (Wang and Szaniszlo, 2000; Ye and Szaniszlo, 2000). To further investigate the effects of *WdSTUA* on filamentation, the *WdSTUA* gene was deleted by replacement from the Hf1 strain. Because our subsequent studies revealed that the phenotypes of several resulting mutants were identical, only the phenotypic comparisons of the wild type with the Hf1*wdstua*Δ*1* mutant are described below. On YPDA at 37°C, the colonies of Hf1 had more convoluted surfaces than those of the wild type and *wdstua*Δ*1A*, which was not particularly affected by deletion of *WdSTUA* (Figure 3.11A). However, culture of Hf1*wdstua*Δ*1* on PDA agar medium at 25°C showed that *WdSTUA* deletion in Hf1 obviously reduced filamentous growth (Figure 3.11B). In contrast to deletion of *WdSTUA* in wild type, deletion of *WdSTUA* in Hf1 still allowed the production of some hyphae, suggesting that *WdSTUA* only influenced one hyphal developmental pathway among a number that may be triggered in Hf1 upon shift to 37°C. Slide culture observations showed that both Hf1 and Hf1*wdstua*Δ*1* produced conidia on PDA at 25°C, although Hf1 produced longer and more abundant conidiophores than the Hf1*wdstua*Δ*1* mutant (Figure 3.11C). These results demonstrated that Hf1*wdstua*Δ*1* strain retained the ability to produce hyphae and conidia.

3.37 Ectopic expression of *WdSTUA* in *W. dermatitidis*

For the ectopic expression study, the previously developed color-selectable and site-specific integrative transformation system for *W. dermatitidis* was used (Ye et al., 1999; Ye and Szaniszlo, 2000). This transformation system uses the nonessential *WdPKS1* genome locus, which encodes a polyketide synthase in the melanin biosynthesis pathway, as the integrative target site for the ectopic expression of a gene. The disruption of *WdPKS1* produces white phenotype, which helps selection. More importantly, the *WdPKS1* deletion does not affect morphology beyond the loss of pigmentation. The *WdSTUA* gene was under the control of *glaA* promoter, which is induced by maltose in *A. niger*. Because the expression vector pYEX303 has an *hph* marker, another set of *wdstua*Δ mutants was made with the *sur* marker for these studies by a one-step gene replacement strategy: the resulting mutants had the same phenotypes as the *wdstua*Δ mutants made with the *hph* marker (data not shown). Transformations with pYEX303 and pYEX303-*WdSTUA* of the wild type and the *wdstua*Δ*2* (*wdstua*::*sur*) strain produced *wdpks1*Δ, which acted as the white, wild-type control, *wdpks1*Δ*WdSTUA,* *wdpks1*Δ*wdstua*Δ, which acted as the white *wdstua*Δ control, and *wdpks1*Δ*wdstua*Δ*WdSTUA*. PCR evaluations with specific primers showed that *wdpks1*Δ produced a 123-bp band from the wild-type *WdSTUA* locus, *wdpks1*Δ*wdstua*Δ did not produce any band, *wdpks1*Δ*WdSTUA* generated a 123-bp *WdSTUA* band and a 68-bp band from the ectopic expressed *WdSTUA*, and *wdpks1*Δ*wdstua*Δ*WdSTUA* had only a 68-bp ectopic expressed *WdSTUA* band (Figure 3.12A).

Preliminary observations of the numerous transformants derived of each type had identical phenotypes. Therefore, the figures presented below only show results for *wdpks1*Δ*-1, wdpks1*Δ*WdSTUA-1, wdpks1*Δ*wdstua*Δ*-1* and *wdpks1*Δ*wdstua*Δ*WdSTUA-1*. When *WdSTUA* was expressed in the *wdpks1*Δ*wdstua*Δ background and cultured on the *glaA* induction medium YPMA at 37°C, the convolutions in the colony surfaces of this mutant were eliminated (data not shown). *WdSTUA* expression in *wdpks1*Δ*wdstua*Δ*WdSTUA-1* also eliminated convolutions in the colony surfaces when grown on YPDA at 37°C (Figure 3.12B). Moreover, on PDA at 37°C, the ectopic expression of *WdSTUA* in the white, wild-type background and in the white, *wdstua*Δ background both mostly inhibited hyphal growth (Figure 3.12B). In this case, the *wdpks1*Δ*wdstua*Δ*-1* mutant produced fewer filaments at the colony periphery than wild type, and the two ectopic expression strains produced the least filamentation. This inhibition of morphotype transition compared to the wild type was also observed when the strains were cultured on PDA at 25°C (Figure 3.12B).

Northern analysis showed that the *WdSTUA* RNA levels in *wdpks1*Δ*WdSTUA-1* and *wdpks1*Δ*wdstua*Δ*WdSTUA-1* were higher than the control *WdSTUA* RNA levels in *wdpks1*Δ when cells were cultured in PDB and YPDB at 25^{o}C and 37^{o}C (Figure 3.12C). Wild-type *WdSTUA* locus produced transcripts about 3 kb. Ectopic *WdSTUA* under the control of *glaA* promoter-terminator mainly produced a transcript about 2 kb, due to the reduced sizes of the 5' and 3'UTR.

The results from these experiments showed that the deregulation of *WdSTUA* in *W. dermatitidis* strongly repressed filamentous growth. In contrast, the ectopic expression of the amino-terminal half of WdStuAp had no effect (data not shown), suggesting that full length WdStuAp is required for its function in *W. dermatitidis*.

3.38 Heterologous expression of *WdSTUA* in *S. cerevisae*

The expression of some transcription factors, including Phd1p and Efg1p, can induce pseudohyphal growth in *S. cerevisae* (Chua et al., 2006; Rottmann et al., 2003). To test whether WdStuAp might also induce pseudohyphal growth in *S. cerevisae*, *WdSTUA* cDNA was cloned into the *S. cerevisae* expression vector pYES2 and transformed into MR12 strain, which contains a *FLO11*-promoter::*lacZ* reporter construct (Rottmann et al., 2003). Compared with MR12 carrying the pYES2 vector without an insert, MR12 containing pYES2-*WdSTUA* induced pseudohyphal growth, the formation of rougher colonies, and the formation of colonies more attached to the agar medium (Figure 3.13). The expression of the amino-terminal half of WdStuAp had no function (data not shown). Because in *S. cerevisiae*, *FLO11,* which encodes a cell wall glycosylphosphatidylinositol (GPI) protein, is required for pseudohyphal growth (Halme et al., 2004), β-galactosidase assays were used to show that the *FLO11-lacZ* activity of the pYES2-*WdSTUA* strain was induced to high levels compared to that of the pYES2 strain (1.3 vs 0.17 Miller units). Furthmore, a microarray analysis was used to show that a number of genes were significantly regulated (Figure 3.14, Appendix B), including *PGU1,* which is up-regulated 1.6, 1.8 and 1.7 folds, respectively, in three biological repeats. *PGU1* has

been reported to be up-regulated from the microarray assays of *S. cerevisae* filamentous pathway (Borneman et al., 2006; Madhani et al., 1999). *PGU1* encodes a polygalacturonase associated with pectin degradation, which facilitates fungal infections to plant hosts. Heterologous expression of the amino-terminal half of WdStuAp did not show these effects. Collectively, these results indicated that the expression of *WdSTUA* activated the pseudohyphal growth pathway in *S. cerevisae*.

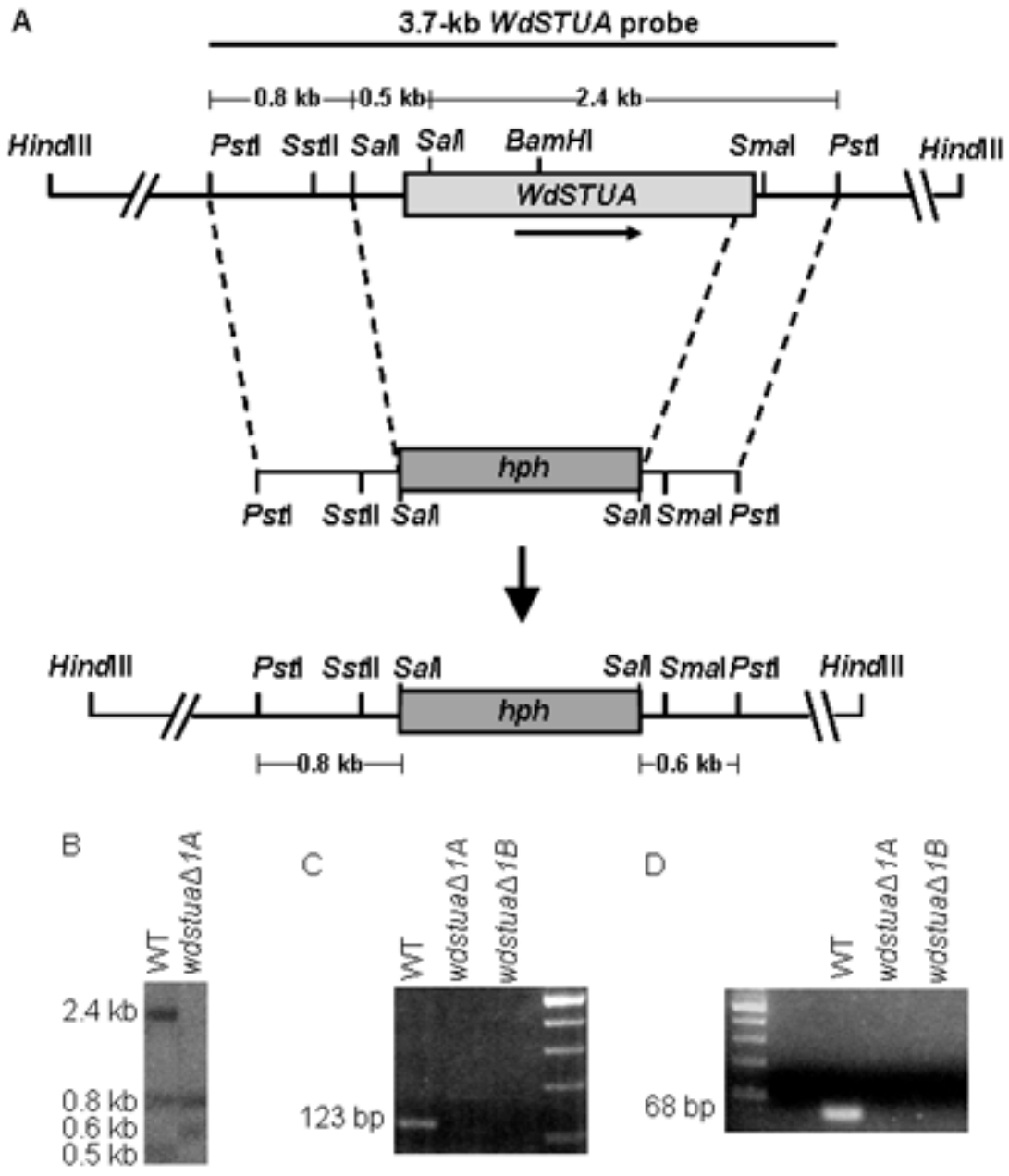

A
3.7-kb WdSTUA probe
0.8 kb
0.5 kb
2.4 kb
HindIII
PstI
SstII
SalI
SalI
BamHI
SmaI
PstI
HindIII
WdSTUA
hph
PstI
SstII
SalI
SalI
SmaI
PstI
HindIII
PstI
SstII
SalI
SalI
SmaI
PstI
HindIII
hph
0.8 kb
0.6 kb
B
WT
wdstuaΔ1A
2.4 kb
0.8 kb
0.6 kb
0.5 kb
C
WT
wdstuaΔ1A
wdstuaΔ1B
123 bp
D
WT
wdstuaΔ1A
wdstuaΔ1B
68 bp

FIGURE 3.5. DISRUPTION OF THE *WDSTUA* GENE BY A ONE-STEP GENE REPLACEMENT METHOD. (A). Strategy for disruption. A 0.8-kb *Pst*I-*Sal*I fragment corresponding to the 5' region of *WdSTUA* and a 0.6-kb PCR amplified 3' region of *WdSTUA* were constructed to flank the *hph* marker. This fragment was transformed into *W. dermatitidis* to produce a recombination with genomic DNA at the 5' and 3' regions. (B). Southern analysis. Genomic DNA of the wild-type strain and disruption strain *wdstua*Δ*1A* was digested with *Pst*I and *Sal*I and electrophoresed on a 0.8% agarose gel. DNA was hybridized with a 3.7-kb *Pst*I-*Pst*I *WdSTUA* probe. The sizes of the hybridization bands are indicated. (C) Analysis of *WdSTUA* deletions by PCR. Genomic DNA of wild type (WT), *wdstua*Δ*1A,* and *wdstua*Δ*1B* was amplified by PCR with primers WSF6 and WSR6. (D) The *WdSTUA* deletion in the mutant was confirmed by RT-PCR. RNA from WT, *wdstua*Δ*1A,* and *wdstua*Δ*1B* was used for the RT-PCR with primers WSF6 and WSR6.

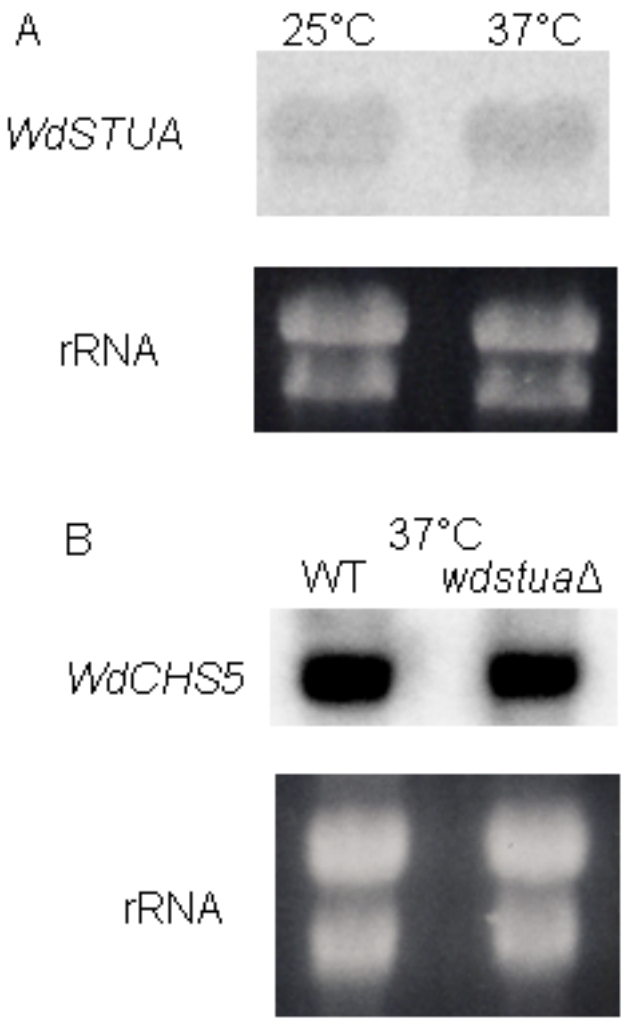


FIGURE 3.6. NORTHERN ANALYSIS OF *WDSTUA* AND *WDCHS5* EXPRESSION. (A) Northern analysis for *WdSTUA* transcript level. Total RNA was prepared from wild-type cells grown in YPDB at 37°C or 25°C for 24 h. 15 µg RNA was loaded and hybridized with a *WdSTUA* probe. (B) Northern analysis for *WdCHS5* transcript level in wild-type strain and *wdstua*Δ*1A* strain. Total RNA was prepared from cells grown in YPDB at 37°C for 24 h. 15 µg RNA was loaded and hybridized with a *WdCHS5* probe.

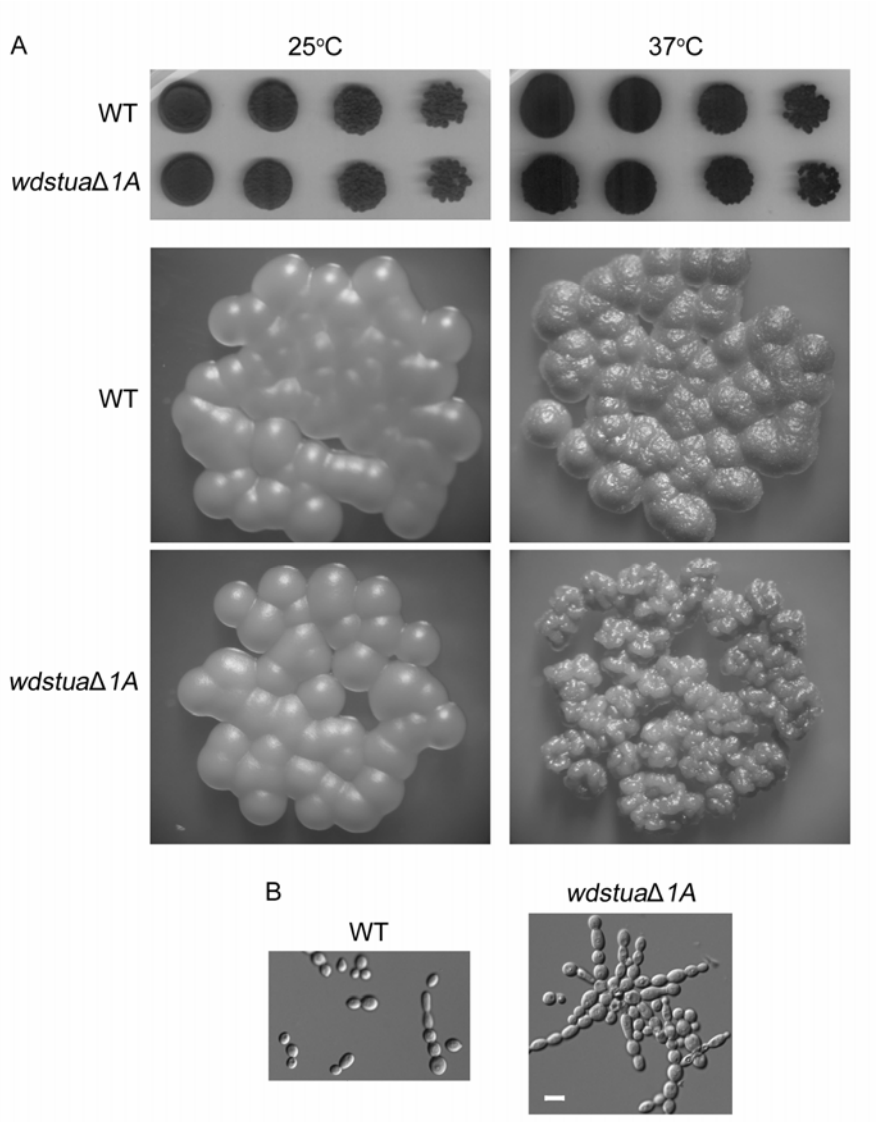


FIGURE 3.7. EFFECTS OF *WDSTUA* DISRUPTION ON COLONIAL PHENOTYPES AT 25°C AND 37°C YPDA. (A) Cells were spotted with 10^5, 10^4, 10^3, and 10^2 cells on YPDA and then incubated at 25°C and 37°C. After 4 days of incubation, the plate was documented and colony morphology of the spots of 10^2 cells was photographed through a dissecting microscope. (B) The wild type and *wdstuaΔ1A* were cultured with shaking in YPDB at 37°C. Late log-phase cells were visualized with a compound light microscope and then photographed. Note that *wdstuaΔ1A* produced more hyphal-like aggregates. The scale bar represents 10 μm and is applicable to each photograph in figure B.

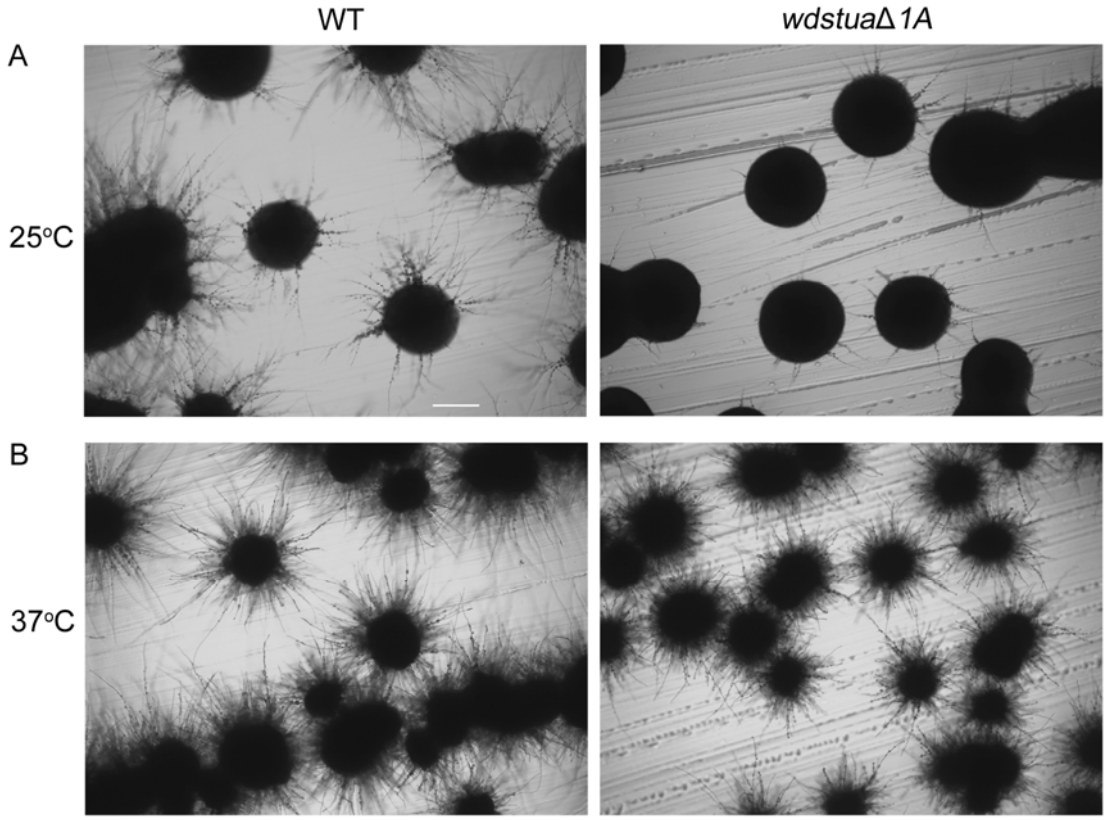


FIGURE 3.8. THE *WDSTUA* DELETION AFFECTED *W. DERMATITIDIS* FILAMENTOUS GROWTH ON PDA AT 25°C. The wild-type and *wdstuaΔ1A* strains were streaked on PDA and incubated at 25°C and 37°C. The filamentous growth at the colony edges was visualized with a compound light microscope and the photographs were taken after 4 days of incubation. The scale bar equals 0.2 mm and is applicable to each photograph in the figure.

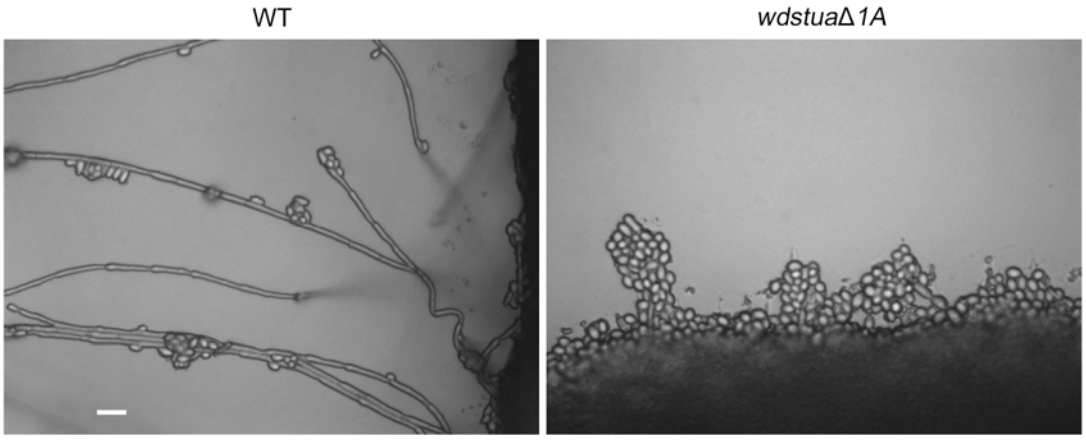


FIGURE 3.9. THE *WDSTUA* DELETION BLOCKED AERIAL HYPHAL GROWTH AND CONSEQUENTLY CONIDIOGENESIS. (A) The wild type and *wdstuaΔ1A* were inoculated on PDA in slide cultures and incubated at 25°C. Growth at the edge of the medium and protruding into the air space between the slide and cover slip was visualized with a compound light microscope and photographed after two weeks of incubation. Infrequently, *wdstuaΔ1A* formed aerial filaments at 25°C, which were very abnormal. The scale bar represents 10 µm is applicable to each photograph in the figure.

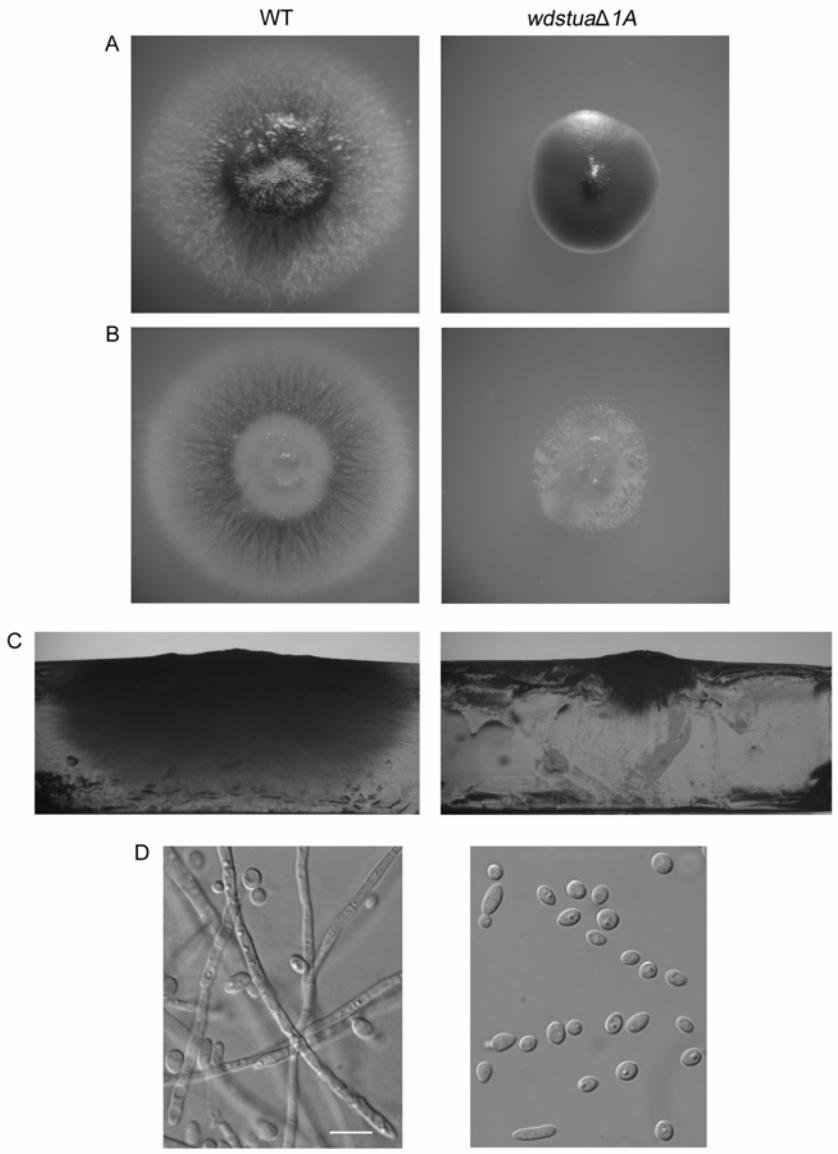


FIGURE 3.10. THE *WDSTUA*Δ MUTANT WAS DEFECTIVE IN INVASIVE GROWTH. (A) Wild type and *wdstua*Δ*1A* cells were spotted on PDA and incubated at 25°C. After incubation for 8 days, colonies were visualized with a dissecting microscope and photographed. (B) The biomass above the agar was then rinsed away and then the remaining, invasive growth photographed again. (C) Cross sections of the growth that invaded the agar. (D) The morphotypes in the invasive growth visualized with a light microscope. The scale bar is 10 μm and applicable to each photograph in figure D.

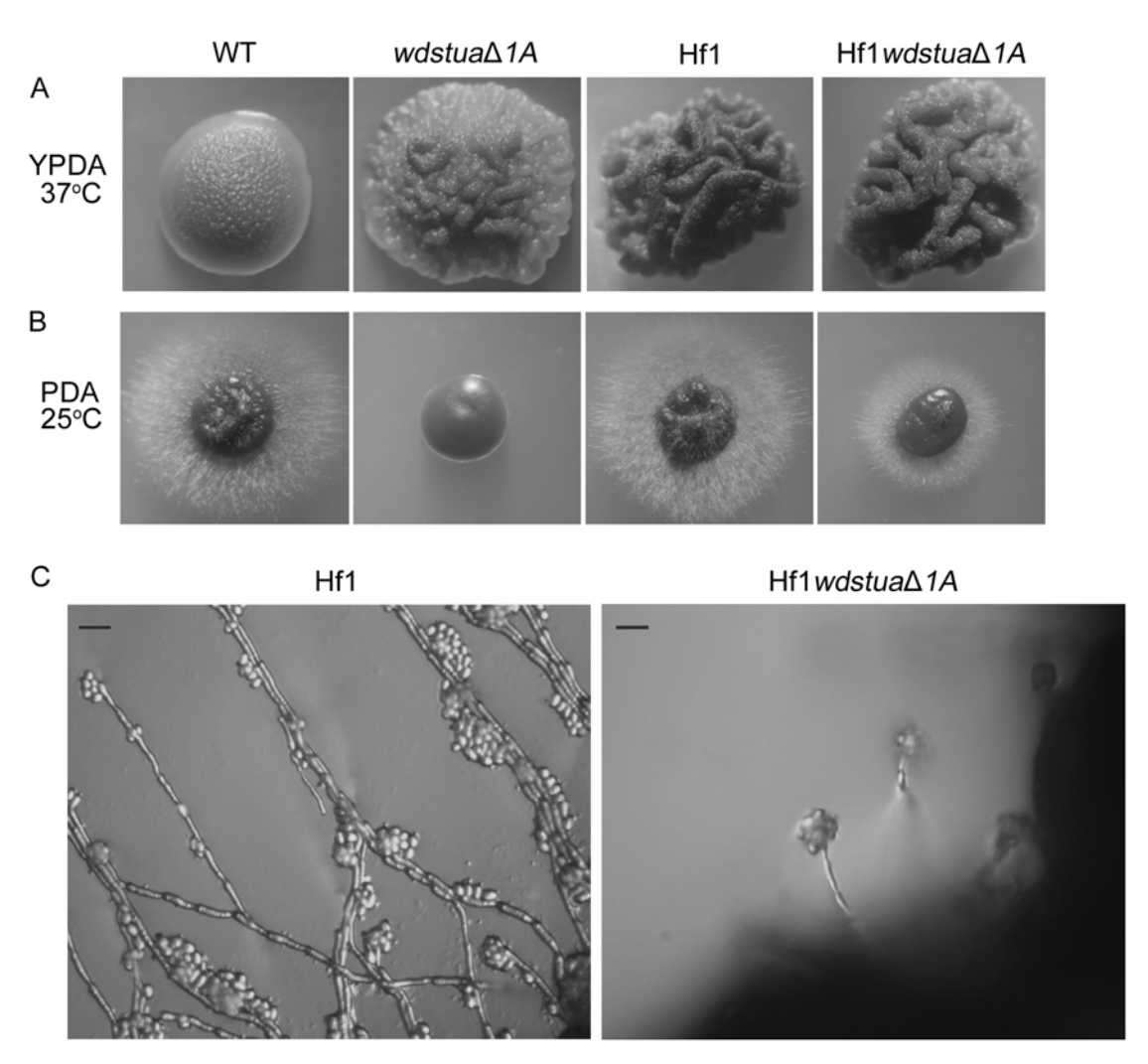
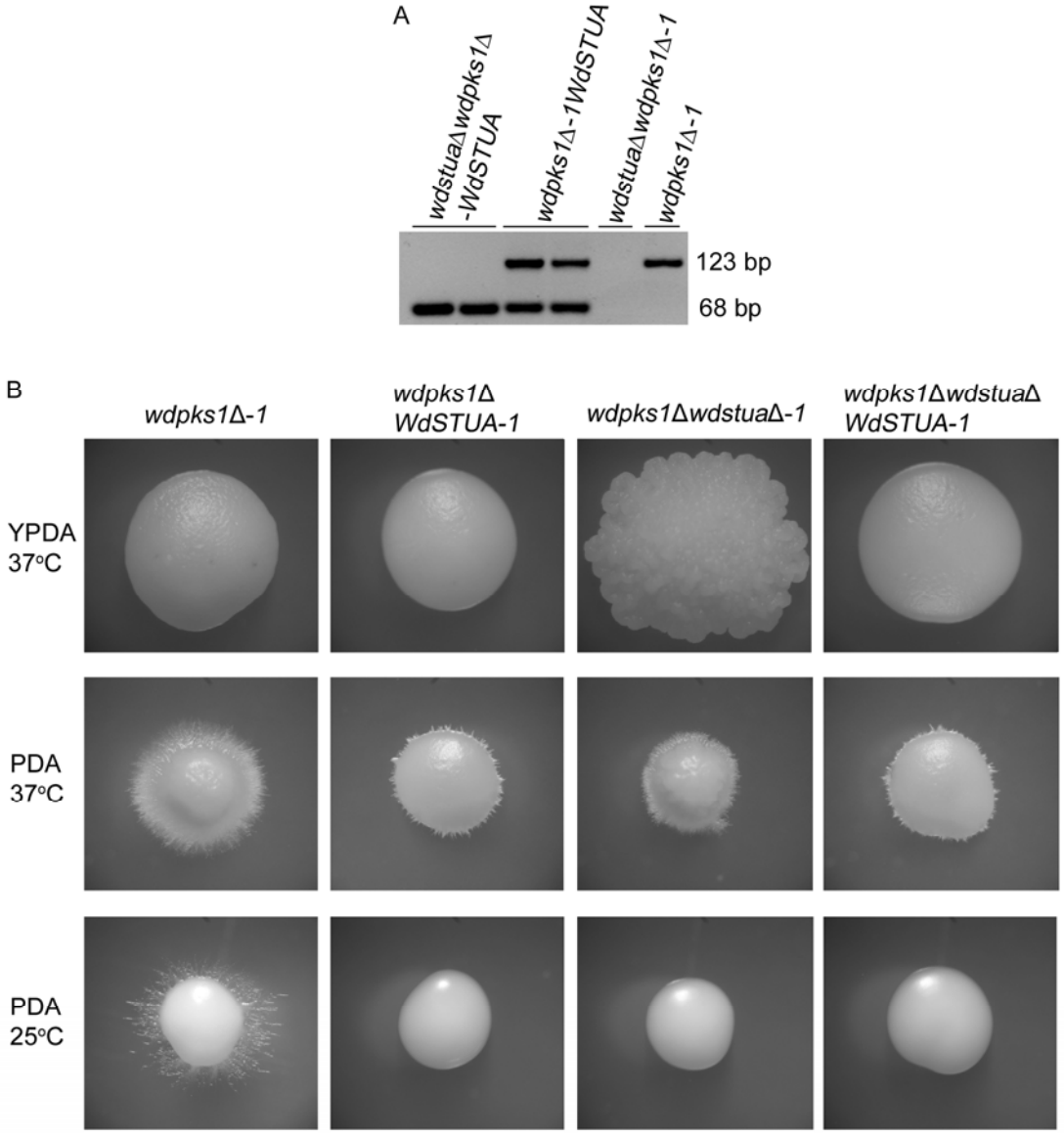


FIGURE 3.11. *WDSTUA* DELETION IN THE HF1 STRAIN INHIBITED FILAMENTOUS GROWTH ON PDA. Wild type, *wdstuaΔ1A,* Hf1 and Hf1*wdstuaΔ1* were grown on YPDA at 37°C for 6 days (A), and on PDA at 25°C for 6 days (B). Hf1 and Hf1*wdstuaΔ1* were inoculated on PDA slide culture and incubated at 25°C. After 2 weeks, conidiophores and conidia were visualized with a light microscope. The scale bars are 10 μm (C).


A
wdstuaΔwdpks1Δ -WdSTUA
wdpks1Δ-1WdSTUA
wdstuaΔwdpks1Δ-1
wdpks1Δ-1
123 bp
68 bp
B
wdpks1Δ-1
wdpks1Δ WdSTUA-1
wdpks1ΔwdstuaΔ-1
wdpks1ΔwdstuaΔ WdSTUA-1
YPDA 37°C
PDA 37°C
PDA 25°C

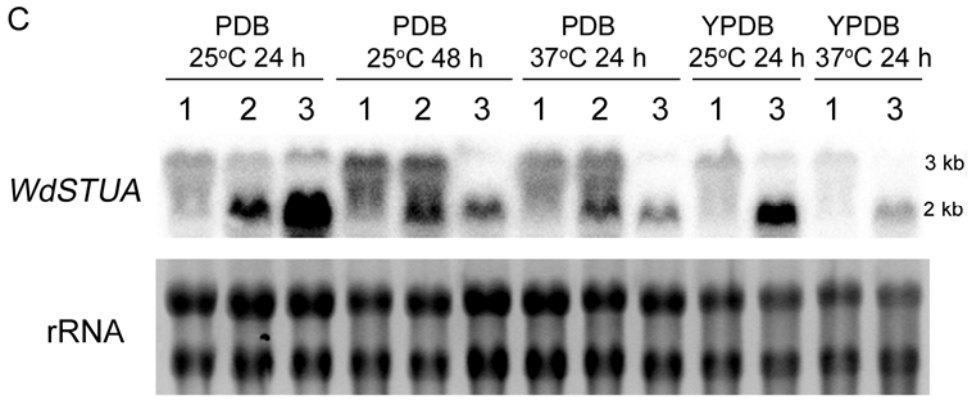


FIGURE 3.12. ECTOPIC EXPRESSION OF *WDSTUA* IN *W. DERMATITIDIS*. (A) PCR amplification of genomic DNA of *WdSTUA* mutation strains with primers WSF6 and WSR6. A 123 bp band was amplified from *WdSTUA* genomic DNA, and a 68 bp band was amplified from *WdSTUA* cDNA. (B) *WdSTUA* mutation strains were grown on YPDA at 37°C, PDA at 37°C and PDA at 25°C. After 5 days of incubation, colonies were photographed through a dissecting microscope. (C) Northern analysis of *WdSTUA* expression of *wdpks1*Δ*-1* (#1)*, wdpks1*Δ*WdSTUA-1* (#2) and *wdpks1*Δ*wdstua*Δ*WdSTUA-1* (#3) grown in PDB and YPDB at 25°C and 37°C. After the hours indicated, total RNA was extracted from the cells. 30 μg RNA was loaded and hybridized with a *WdSTUA* probe. Ethidium-bromide stained rRNA was as a control for relative RNA amounts.

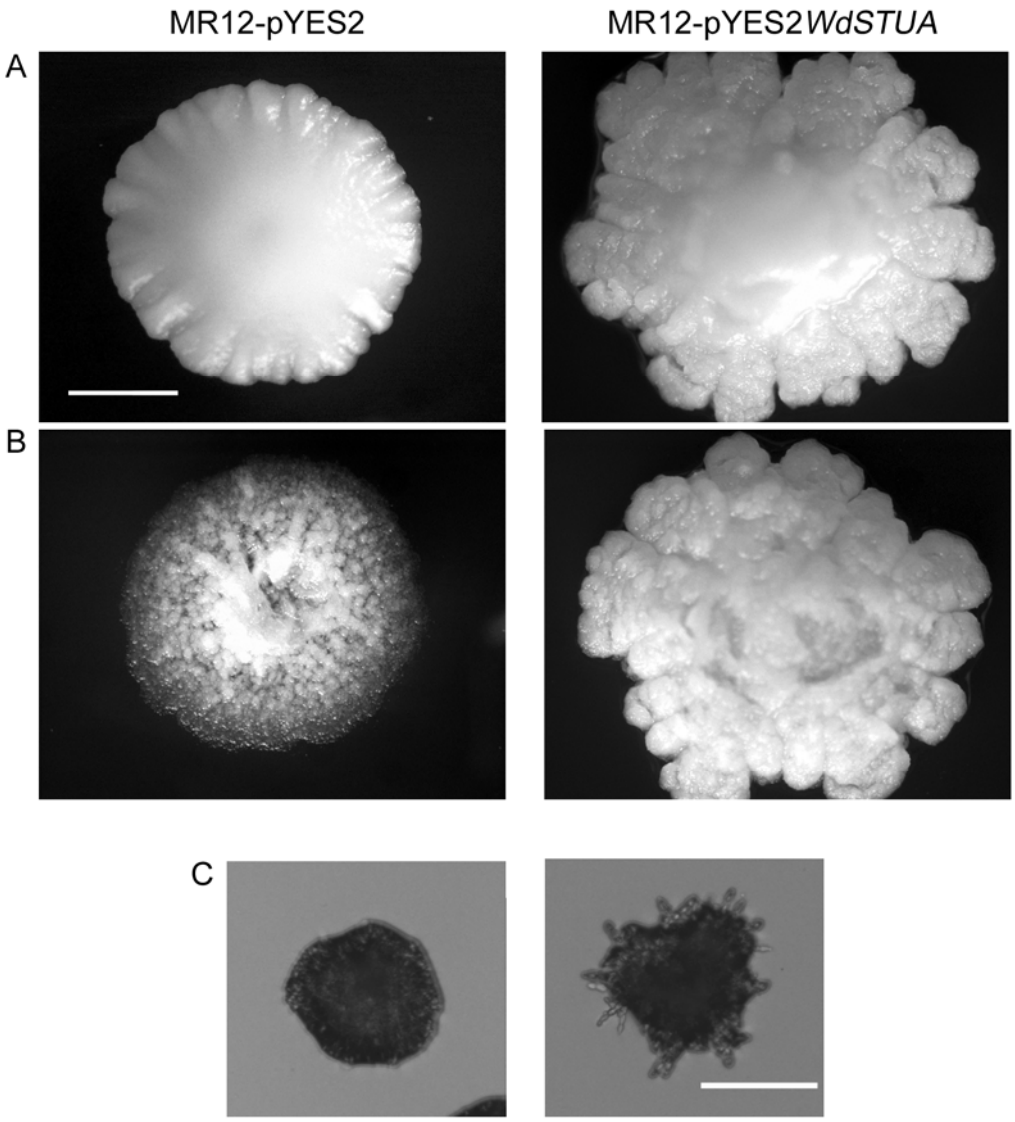

MR12-pYES2
MR12-pYES2*WdSTUA*
A
B
C

FIGURE 3.13. EXPRESSION OF *WDSTUA* IN *S. CEREVISAE*. (A) The MR12-pYES2 empty vector strain and the MR12-pYES2*WdSTUA* expression strain were spotted on SLARA medium and incubated at 30°C. Note that the MR12-pYES2*WdSTUA* colony had a rougher boundary than that of MR12-pYES2. After 6 days of incubation, the colonies were visualized with a light microscope fitted with a 2.5 x objective and photographed. (B) After rinsing with tap water, colonies were photographed again. Note the colony of MR12-pYES2*WdSTUA* adhered more to the agar than did that of MR12-pYES2. The scale bar in figure A represents 1 mm and is applicable to all the photographs in figure A and B. (C) The MR12-pYES2 empty vector strain and the MR12-pYES2*WdSTUA* expression strain were streaked on SLARA medium and incubated at 30°C. MR12-pYES2*WdSTUA* stimulated more filamentous growth compared with MR12-pYES2. After 2 days of incubation, representative colonies were visualized with a compound light microscopy fitted with a 10 x objective and photographed. The scale bar in figure C represents 0.1 mm and is applicable to each photograph in figure C.

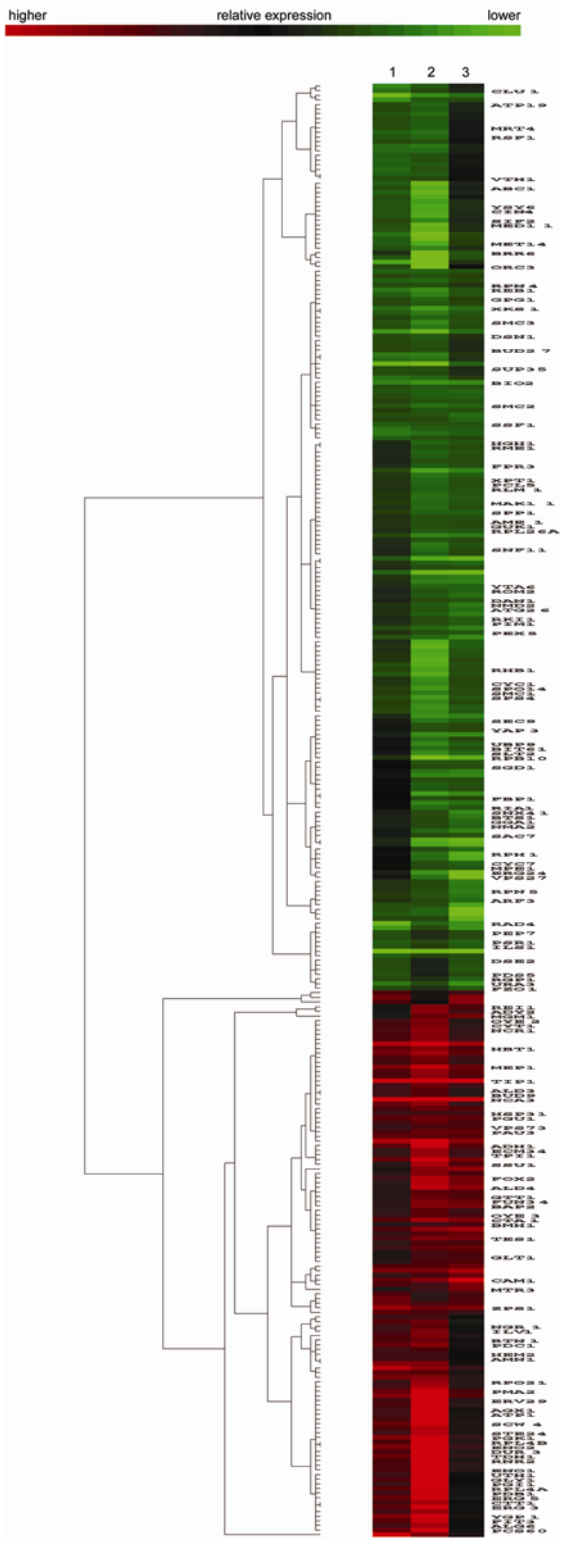


FIGURE 3.14. EXPRESSION PROFILES OF REGULATED GENES BY THE PYES2-*WDSTUA* EXPRESSION STRAIN VS. THE PYES2 VECTOR ALONE STRAIN. The data is clustered by Cluster program and the clustering result is displayed by TreeView program. Three biological repeats are indicated by column 1, column 2, and column 3. Full list of regulated genes and their regulated levels can be found in Appendix B.

3.4 DISCUSSION

Known or suspected Ascomycota species are often divided somewhat arbitrarily into two groups, those that are called molds or filamentous fungi and those called yeasts. Fungi in the first group must generally reproduce vegetatively by fragmentation or by the production of asexual spores called conidia, whereas those in the second group tend to be single cell fungi that grow reproductively by budding or fission. However, many species in both groups are vegetatively dimorphic or polymorphic and thus able to express growth in more than one manner. For example, although the predominant morphotype of *W. dermatitidis* is a yeast cell, it is in actuality a filamentous, conidiogenous mold. Although a number of environmental factors have been identified that trigger the transition of the yeast morphotype of *W. dermatitidis* to one of its alternate morphotypes (Szaniszlo, 2006), nothing is known about the roles played by any transcription factor in controlling those transitions.

After the *WdSTUA* gene of *W. dermatitidids* was identified and cloned by a degenerate PCR method, analyses showed that its APSES DNA-binding domain was well conserved in fungi, implying it evolved early and has remained relatively unchanged since. Similar with its homologs in other filamentous fungi (Borneman et al., 2002; Miller et al., 1992; Ohara and Tsuge, 2004), *WdSTUA* has three introns near and at the region encoding the 78-aa APSES domain (Figure 3.2, 3.3). Multiple alignment of WdStuAp with orthologs of closely related filamentous fungi showed several conserved sequence fragments (Figure 3.4): First, amino acid residues 122 to 231, a region that includes the

APSES domain; second, residues 308 to 321, a region rich in proline, serine and threonine; third, residues 359 to 371, a second region also rich in proline, serine and threonine; fourth, residues 577 to 581, a cluster of charged amino acids; fifth, residues 598 to 601, a region of all positive amino acids that contains a predicted nuclear localization signal. These sequences may play important roles in WdStuAp interactions with other proteins and in regulation. When the amino-terminal half of WdStuAp was expressed in *W. dermatitidis* and *S. cerevisiae*, it was found not to be functional, even though it contained an APSES DNA binding domain. This suggested that the full length WdStuAp is required for its function (data not shown). In contrast with the finding that *PmSTUA* transcripts are only detected in conidiogenous cells (Borneman et al., 2002), our Northern analysis showed that *WdSTUA* was expressed at similar levels in vegetative growth during culture in YPDB and PDB at 25°C and 37°C (Figure 3.6, 3.12C). Also, the *WdSTUA* RNA level was found to be similar when cells were cultured on PDA for 6 da (data not shown). Therefore, the expression patterns for *WdSTUA* is more like the situation with *FoSTUA* and *AnSTUA* transcripts, which are also expressed during both vegetative growth and conidiation (Miller et al., 1991; Ohara and Tsuge, 2004).

A functional characterization of WdStuAp was possible because the deletion of *WdSTUA* from the *W. dermatitidis* genome was not a lethal event. This allowed for the production of numerous *wdstua*Δ knock out mutants not only in the wild-type strain, but also in a strain defective in melanin biosynthesis (*wdpks1*Δ) and in a so-called hyphal-form mutant strain (Hf1). The latter is temperature sensitive and grows on rich media as a yeast like wild-type at 25°C, but forms mostly hyphae at 37°C in the same media. In

spite of these different genetic backgrounds, all of the mutants that were derived having a *WdSTUA* deletion expressed the same general characteristics, with the obvious exceptions that the *wdpks1*Δ*wdstua*Δ double mutants were albino and the Hf1*wdstua*Δ double mutants tended to produce more hyphae. Unfortunately, for unknown reasons, our attempts to disrupt *WdSTUA* in our ts Mc3 (*WdCDC2*) mutant, which converts to sclerotic cells and sclerotic bodies at 37°C, were unsuccessful. Therefore, we investigated whether the *WdSTUA* deletion affected the production of the isotropic morphotypes and their ability to proliferate by a slow fission under MCDB pH 2.5 conditions (Wang and Szaniszlo, 2000). In the manner of the wild type, *wdstua*Δ still produced sclerotic cells, planate cells and sclerotic bodies (data not shown). This is in agreement with a previous report that yeast growth by fission in *P. marneffei* is not affected by *PmSTUA* deletion, and the fact that the genome of the fission yeast *S. pombe* does not have an APSES transcription factor gene (Borneman et al., 2002).

The most striking initial observation suggesting that deletion of *WdSTUA* might affect morphotype transitions between yeast and hyphae in *W. dermatitidis* was our finding that the *wdstua*Δ deletion strains produced colonies with severely convoluted surfaces compared with those of the wild type during culture in rich YPDA medium at 37°C (Figure 3.7). Colonies of the *wdstua*Δ mutants were less convoluted than those of the ts Hf1 strain at 37°C (Figure 3.11A), suggesting that WdStuAp is associated with partial expression of this morphological trait. Among ascomycetous yeast species, such correlations between colony morphology changes and morphotype changes are best known in *C. albicans*, such as those associated with white-opaque colony morphology

switching of the WO-1 strain (Soll, 1992). *EFG1* deletion strains of this pathogen display opaque colonies that contain elongated yeast (Sonneborn et al., 1999b), and it is documented that Efg1p is required for a subset of characteristics associated with this colony switching event (Srikantha et al., 2000). In *S. cerevisiae,* convoluted colony morphology is similarly reported to be related to the polarized growth of yeast cells and to cell wall adhesin proteins (Vopalenska et al., 2005). It was suggested that adhesion prohibited yeast cells from being exposed to the environment (Verstrepen and Klis, 2006). For the pathogenic fungus *W. dermatitidis*, we presume that this could lead to less contact with the host and thus affect infectivity.

WdSTUA is not only required for smooth yeast colonial growth on rich media, but also for vigorous aerial and invasive hyphal growth in a number of hyphae-inducing media, as demonstrated by the observations that deletion of *WdSTUA* produced severe defects in these properties compared with the wild type (Figure 3.8, 3.9, 3.10). For aerial and invasive hyphal growth to occur, some cell wall proteins are required. For example, hydrophobins are cell wall proteins reported to be necessary for aerial hyphal growth, which serve as surface activators to overcome surface tensions and to provide hydrophobic surfaces on aerial hyphae to prevent desiccation (Elliot and Talbot, 2004). In *S. cerevisae,* which does not produce aerial hyphae, *FLO11* is required for pseudohyphal growth at the colony periphery and cell adhesion to substrates (Verstrepen and Klis, 2006). In *C. albicans*, which also does not produce aerial hyphae, many cell wall genes are identified as hyphal-phase specific and are required for biofilm formation (Liu, 2001; Nobile and Mitchell, 2006). Possibly, *WdSTUA* also activates similar cell

wall proteins. In addition, *WdSTUA* appears to be part of a network of pathways needed to produce filaments during development. Unlike growth at 25°C on PDA, *wdstua*Δ produces more filaments at 37°C (Figure 3.8). Also, a *WdSTUA* deletion in the ts hyphal-form mutant strain Hf1 still permits filament production to some degree even at 25°C (Figure 3.11). Elevation of temperature and mutations in Hf1 might stimulate other parallel pathways to suppress the *wdstua*Δ phenotype.

In *A. nidulans, A. fumigatus*, and *P. marneffei,* StuAp proteins are necessary for the development of metulae and phialides, but not conidia (Borneman et al., 2002; Miller et al., 1991; Sheppard et al., 2005). In *F. oxysporum*, FoStuAp is required for the development of conidiophores, but neither for phialides nor for conidia (Ohara and Tsuge, 2004). By comparison, because WdStuAp is required for the development of aerial hyphae under most conditions, *W. dermatitidis* is inhibited in conidiophore and conidium production when this protein is absent. The one exception in our experiments to this trend occurred when aerial hyphal growth was induced in the Hf1*wdstua*Δ strain cultured at 25°C: this ts strain still generated conidiophores and conidia characteristic of *W. dermatitidis*. The reason for these apparent diversities among different species possibly relates to the functions of the products of additional genes, such as *BRLA* and *ABAA*, which are known to influence conidiogenesis in other fungi. For example, *BRLA* and *ABAA* are central genes required for conidiation in *A. nidulans*, and AnStuAp is required for spatial distribution of AnBrlAp and AnAbaAp in that fungus (Adams et al., 1998). However, genome sequencing completed *Fusarium graminearum* is searched to contain no *BRLA* and *ABAA* homologs, and *S. cerevisiae* and *C. albicans* have no *BRLA*

homologs (Ohara and Tsuge, 2004; Vallim et al., 2000). Unfortunately, the situation in *W. dermatitidis* is yet unknown. Attempts to amplify an *ABAA* homolog in *W. dermatitidis* have so far failed, indicating possibly that this fungus may also be devoid of this gene. Finally, it is important to note that APSES transcription factors, which are mostly reported to be involved in the regulation of the cAMP-PKA pathway, have also been shown to be involved in pigment production (Brakhage and Liebmann, 2005). In *A. fumigatus*, a pigment derived from precursors also found in the melanin biosynthetic pathway of *W. dermatitidis* gives conidia a green appearance in wild-type strains, yet *afstua*Δ mutants appear white due to the defect in pigment production (Sheppard et al., 2005). However, our experiments did not show dramatic effects of the deletion of *WdSTUA* on melanin deposition in this *W. dermatitidis*, thus providing further evidence that *WdSTUA* mainly has effects related to yeast-filamentous transitions in this black fungus.

Among the APSES factors studied, growth under hypoxic conditions has only been reported with respect to Efg1p of *C. albicans*. Under limited oxygen at temperatures lower than 37°C, an *EFG1* null strain is more filamentous than the wild type, and Efg1p is required to regulate different sets of genes (Setiadi et al., 2006). In *W. dermatitidis,* under hypoxic growth conditions (embedded in YPSA), *wdstua*Δ did not produce more filaments than wild type (data not shown), indicating WdStuAp has a divergent regulatory effect compared with that of Efg1p.

Expression of *WdSTUA* under the control of the *glaA* promoter in the wild type background and in the *WdSTUA* deletion background repressed filamentous growth. The convoluted growth of *wdstua*Δ on YPDA at 37°C was reversed (Figure 3.12B). The filamentous growth on PDA was reduced (Figure 3.12B). Also, when cells were grown embedded in YPSA, the filamentation was dramatically repressed by the ectopic expression of *WdSTUA* (data not shown). In addition, cells grown on PDA under coverslips observed with microscope showed that hyphal cells were largely decreased in the *WdSTUA* ectopic expression strains (data not shown). The results from these experiments all showed that the ectopic expression of *WdSTUA* in *W. dermatitidis* strongly represses filamentous growth. These findings are in agreement to those obtained with those with *C. albicans*, which documented that *EFG1* overexpression causes a switch from the opaque phase phenotype to the white phase phenotype (Sonneborn et al., 1999b), and that *EFG1* overexpression represses hyphal growth and produces pseudohyphal development instead (Stoldt et al., 1997; Tebarth et al., 2003). It has been found that after initiation of hyphal growth, *EFG1* expression is immediately repressed at the beginning of the hyphal elongation process (Stoldt et al., 1997; Tebarth et al., 2003). Northern analysis showed that *WdSTUA* RNA levels were increased in the ectopic expression strains compared with the control strains (Figure 3.12C), suggesting that normal level *WdSTUA* expression is required for the normal *W. dermatitidis* hyphal cell differentiation. It is interesting to note that in contrast to the effects of expression of *WdSTUA* in *W. dermatitidis* and overexpression of *EFG1* in *C. albicans*, overexpression of *PHD1* strongly induces filamentous growth in *S. cerevisiae* (Gimeno and Fink, 1994).

WdSTUA expression in *S. cerevisae* triggered peudohyphal growth (Figure 3.13), and activated the *FLO11* promoter. *WdSTUA* expression effects rely on the other genes in *S. cerevisiae.* Genome-wide transcript profiles showed many genes of *S. cerevisiae* involved in development and metabolism were significantly regulated by WdStuAp (Figure 3.14, Table 3.3, Appendix B), which is consistent with microarray assay results of other APSES factors. Among the genes activated, many were involved in pseudohyphal growth. For example, *PGU1* codes for polygalacturonase associated with pectin degradation, which facilitates fungal infection in plant hosts. *PGU1* has also been reported from the microarray assay of the *S. cerevisae* filamentous pathway (Borneman et al., 2006; Madhani et al., 1999). Also, upregulated were some cell wall genes, which might enhance cell strength and substrate penetration. Among these were *TIP1,* which encodes a major cell wall, temperature-shock-inducible GPI-CWP, the absence of which leads to a weakened cell wall having increased sensitivity to calcofluor white and congo red (Fujii et al., 1999), Another is *YGP1,* which encodes a secretary glycoprotein homologous to the sporulation specific Sps100p. Transcription of *YGP1* is induced by nutrient starvation (Destruelle et al., 1994). Two others are *FIT3* and *ZPS1*, which are GPI-CWPs involved in the transportation of iron and zinc, respectively. Another is *SCW4,* which encodes a cell wall protein with glucosidase activity. *scw4scw10* double mutants are defective in mating (Cappellaro et al., 1998). And another yet is *UTH1,* the product of which is a SUN family member, which consists of the homologous glycosylated proteins Sim1p, Uth1p, Nca3p and Sun4p. Among these, Uth1p is localized to both the cell wall and the mitochondrial outer membrane, which may link mitochondrial biosynthesis to cell wall response (Velours et al., 2002).

Expression of *UTH1* is controlled by an oxidative stress response (Bandara et al., 1998), and *UTH1* null mutant strains show growth defects in non-fermentable carbon source utilization and decreased levels of chitin (Lesage et al., 2005; Steinmetz et al., 2002). The *NCA3* gene of the SUN family is also up-regulated.

Numerous genes for fermentation, fatty acid oxidation, and oxidative stress were also activated by the expression of WdStuAp in *S. cerevisiae.* For example, both catalase genes in *S. cerevisiae*, *CTT1* and *CTA1,* were up-regulated. This is consistent with the finding that *AfSTUA* regulates the catalase gene *CAT1* in *A. fumigatus,* as determined by both microarray assay and real-time PCR assay, and supported by the fact that *afstua* mutatnts are more sensitive to H_2O_2 treatment (Sheppard et al., 2005). *GTT1* encodes glutathione S-transferase and is involved in the response to antioxidative stress (Collinson and Grant, 2003), as glutathion (GSH) reacts with reactive oxygen species and plays an important role in antioxidative stress. *OYE2* and *OYE3* encode NADPH dehydrogenase, and loss of *OYE2* displays growth defects on non-fermentable carbon substrates (Steinmetz et al., 2002). Moreover, genes related to nitrogen metabolism were activated, including *ADY2, FUN34* and *MEP1*, which encode ammonium transporters on the cell membrane (Palkova et al., 2002). *ADY2* also transports acetate and is required for meiosis and sporulation (Paiva et al., 2004; Rabitsch et al., 2001). Other membrane transporter genes include *SSU1*and *BAP2*. *SSU1* produces a sulfite transporter, which is related to efflux of toxic compounds (Park and Bakalinsky, 2000). *BAP2* encodes an amino acid permease, which is induced by TOR (Target of Rapamycin) signaling pathway (Peter et al., 2006). Further, genes for protein biosynthesis such as

RPL4B and *RPL4A* were up-regulated, encoding the large ribosomal subunit (Lecompte et al., 2002). Ergosterol is located in the fungal cell membrane, whose function is same as the function of cholesterol in the human cell membrane. Some ergosterol biosynthesis genes (*ERG*3 and *ERG*5) were up-regulated, while *ERG*24 was down-regulated. *BIO2* encodes an enzyme in biotin biosynthesis pathway. *BIO2* was also found to be down-regulated from the microarray assay of *PHD1* overexpression (Borneman et al., 2006). *WdSTUA* may be involved in various cellular processes, and some appeared to up-regulate pseudohyphae, cell wall, fermentation, fatty acid oxidation, and ammonium transport, and protect from oxidative stress. These results support the concept that *WdSTUA* might contribute to fungal pathogenesis by integrating morphogenesis and metabolic responses that overcome host immune response.

Table 3.3. Subsets of *S. cerevisiae* genes regulated by heterologous expression of *WdSTUA**

Categories	Genes
Fermentation	*PGI1, PFK1, TDH1, TPI1, PGK1, ENO1, ENO2, ADH1, PDC1, ALD4*
Oxidative stress	*CTT1, CTA1, GTT1*
Fatty acid β-oxidation	*FOX2, TES1, PDB1*
Membrane transport	*FUN34, MEP1, ADY2, SSU1, BAP2*
Cell wall	*YGP1, SCW4, TIP1, UTH1, NCA3, FIT3, ZPS1*
Morphogenesis	*PGU1, BMH1, HBT1*
Ergosterol biosynthesis	*ERG3, ERG5,* ***ERG24***
Biotin biosynthesis	***BIO2***

* Down-regulated genes are indicated in bold.

In summary, WdStuAp is an important regulator of yeast-hyphal transitions in *W. dermatitidis*. My results document for the first time among conidiogenous fungi that this APSES transcription factor can act both as a positive and negative regulator. My evidence for this conclusion was that at the wild type *WdSTUA* expression level, WdStuAp negatively regulated the filamentous growth on rich media at 37°C and positively regulated filamentous growth on nitrogen poor media at 25°C and that *WdSTUA* overexpression strongly repressed *W. dermatitidis* filamentous growth. Because filamentous growth was more strongly repressed by the overexpression of *WdSTUA* than by its deletion, *WdSTUA* overexpression may affect other pathways that repress the filamentous growth in the complexed filamentous network. However, it is also tempting to suggest that overexpression of *WdSTUA* may have inhibited unknown upstream pathway components that efficiently inhibited the filamentous growth. Because WdStuAp has a bHLH-DNA binding domain and plays positive and negative regulatory roles, in the manner of the myc transcription factor of mammalians, WdStuAp may act in a similar manner of that well studied transcription factor. For example, Myc binds to transcription factor Max to activate target genes (Ferre-D Amare et al., 1993) and Myc binds to transcription factor Miz1 to inhibit another set of genes (Peukert et al., 1997). Similarly, WdStuAp may play its positive and negative regulator roles by forming different heterodimers with different transcription factors as occurred on PDA at 25°C and on YPDA at 37°C. Then when *WdSTUA* is overexpressed, higher levels of WdStuAp may competitively form heterodimers with other transcription factors, leading to more strongly repress the filamentous growth pathways. I anticipate that additional

exploration of the WdStuAp-associated proteins, the pathways that regulate the activity of WdStuAp and the signals that turn on *WdSTUA* expression will help us better understand the mechanisms controlling yeast and hyphal transitions and ultimately their contribution to fungal pathogenesis.

Chapter 4 WdPacCp regulates pH response, salt stress, and morphology in *W. dermatitidis*

4.1 INTRODUCTION

4.11 *PACC/RIM101* genes

In the 1960s, Dr. Gordon Dorn isolated many mutants of *A. nidulans* that affected acid and alkaline phosphatase activity, and some *pacc* mutants were among them (Dorn, 1965). Before the cloning of *PACC*, genetic analysis had shown that the products of *PALA, B, C, F, H,* and *I* are in a pH signal transduction pathway and that the product of *PACC* is directly involved in pH regulation. In 1995, the *PACC* gene was cloned by complementation of a $pacc^c$ mutant (Tilburn et al., 1995).

In 1993, the Mitchell laboratory screened *S. cerevisiae* mutants that decreased the expression of the sporulation reporter gene *IME2-lacZ*, and among the identified mutants, *rim1*Δ (later called *rim101*, regulator of *IME2*), *rim8*Δ, *rim9*Δ, and *rim13*Δ showed similar phenotypes (Su and Mitchell, 1993a). These mutants decrease the expression of *IME1*, which encodes a meiotic transcription factor that activates many meiotic genes, including *IME2* (Su and Mitchell, 1993a). In the same year, *RIM101* was cloned by complementation of the *rim101-1*Δ mutant (Su and Mitchell, 1993b). Rim101p, Rim8p, Rim9p, and Rim13p are the orthologs of PacCp, PalFp, PalIp, and PalBp, respectively. In 1997, Rim20p was subsequently identified as a PalAp homolog from the *S. cerevisiae*

genome (Negrete-Urtasun et al., 1997), and in 2000, Rim21p was cloned as a PalHp ortholog (Treton et al., 2000).

Because the study of the *PACC* gene of *A. nidulans* began earlier and more information is thus available about this gene and its encoded protein, most of the detailed review below is focused on *PACC*.

4.12 Structure of PacCp

PACC encodes 674 aas (Figure 4.1). A DNA-binding domain that contains three Cys_2His_2-type zinc fingers is present at the N terminus of PacCp (Mingot et al., 1999; Tilburn et al., 1995). The binding of the DNA-binding domain and its recognition site, GCCAAG, has been intensely investigated through EMSA, footprinting, mutagenesis, and structural modeling (Espeso et al., 1997; Tilburn et al., 1995). Four PacCp recognition sites are located in the 800-bp region upstream of the initiation codon of *PACC*, suggesting that the expression of *PACC* is auto-regulated by PacCp (Tilburn et al., 1995).

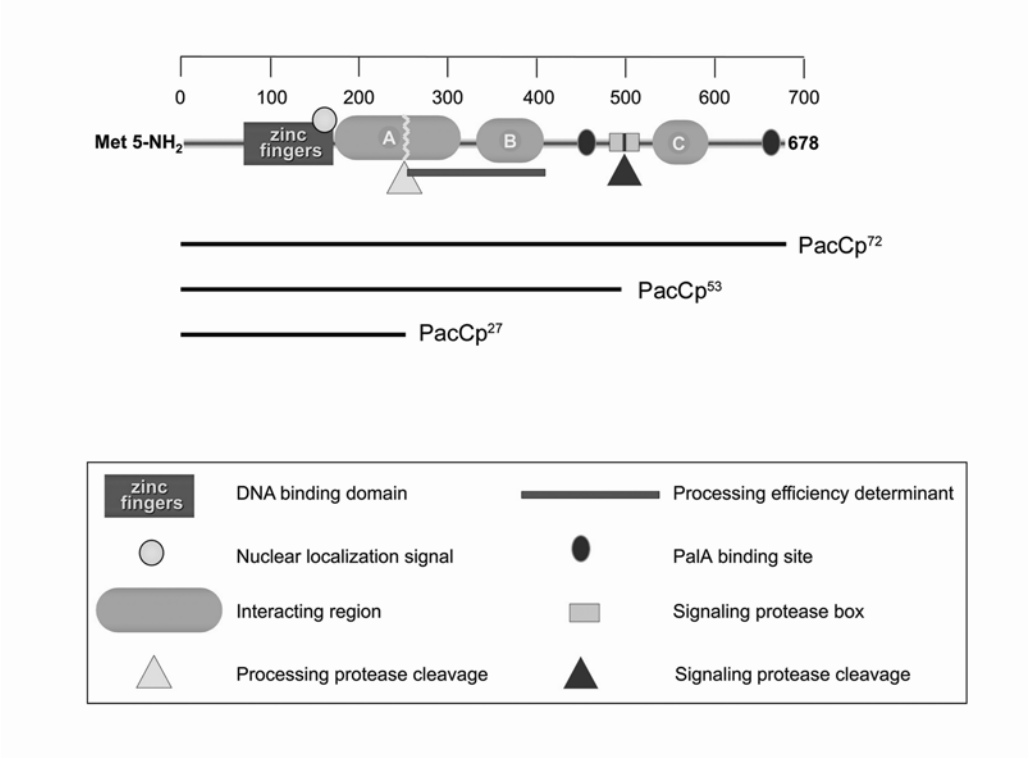


FIGURE 4.1. THE STRUCTURE OF ANPACCP (adapted from Penalva and Arst, 2004).

The full length PacCp is 72 kDa, called $PacCp^{72}$. Studies of mutants show that the deletion of the C terminus 150 aa of PacCp, producing $PacCp^{53}$, causes constitutive activation (*pacc*^{c}) and that a signaling protease box is at residues 493–500 (Diez et al., 2002; Tilburn et al., 1995). EMSA and Western blotting revealed that under acidic conditions or in *pal*Δ mutants (the pH signal pathway is blocked) a $PacCp^{72}$ is predominant, whereas under alkaline conditions or in *pacc*^{c} mutants an N terminal 250 amino acid complex $PacCp^{27}$ is predominant, which indicates that a pH signaling pathway activates a signaling protease to cleave the C terminus of PacCp, yielding $PacCp^{53}$, and then $PacCp^{53}$ was cleavaged to functional form $PacCp^{27}$ by a processing protease that is independent of pH signaling (Mingot et al., 1999; Orejas et al., 1995). This processing protease cleaves within residues 252–254 (Mingot et al., 1999), which is about 90 residues downstream of the zinc finger region.

The sequence upstream of residue 235 decides the specificity of the processing. Also, truncation before residue 333 inhibits processing. In addition, deletion of residues 465–540 and point mutation of L259R, L266F, or L349S makes a PacCp conformational change so that PacCp cannot be processed. Yeast two-hybrid system analysis and immuno-precipitation showed that 150 residues at the C terminus of PacCp (region C in Figure 4.1) interact with the two regions downstream of the DNA binding domain (region A and B in Figure 4.1). When they interact, the conformation of PacCp is in a closed form, and when they do not interact the conformation of PacCp is in an open form. If the C terminus is cleaved away by the pH signaling protease, generating $PacCp^{53}$, the

interaction cannot happen, so the processing by processing protease takes place, yielding $PacCp^{27}$ (Espeso et al., 2000). While $PacCp^{72}$ is not located in the nucleus, $PacCp^{53}$ and $PacCp^{27}$ are. A nuclear localization signal is found in zinc finger 3 (Fernandez-Martinez et al., 2003; Mingot et al., 2001). $PacCp^{27}$ is the predominant form in the nucleus. *RIM101* encodes 628 amino acids. Three Cys_2His_2-type zinc finger motifs are located at the N terminus of Rim101p (Su and Mitchell, 1993b), and the binding site is predicted to be TGCCAAG (Harbison et al., 2004). The only homologous region shared by Rim101p and PacCp is the three C_2H_2 zinc finger DNA-binding domain. In *rim8*Δ, *rim9*Δ, and *rim13*Δ mutants, Rim101p is not cleaved, and expression of a C terminal truncated Rim101p suppresses the deletion effects of *rim8*Δ, *rim9*Δ, and *rim13*Δ under alkaline pH conditions. The Rim101p and PacCp pathways are known to be conserved, with the only difference between them being that Rim101p is activated by one step cleavage, the signaling protease cleavage, whereas PacCp is activated by a two step cleavage process (Li and Mitchell, 1997). When $PacCp^{72}$ is expressed in *S. cerevisiae,* it cannot be cleavaged by the signaling protease, but $PacCp^{53}$ can be cleavaged in *S. cerevisiae* by the processing protease (Mingot et al., 1999).

4.13 The pH pathway that activates PacCp

PacCp is activated in response to elevated pH. PalIp has four trans-membrane (TM) regions, and PalHp has seven (Denison et al., 1998; Negrete-Urtasun et al., 1999). These regions possibly act as the pH sensors in the cell membrane. Among *pal*Δ mutants, *palI*Δ

has a weakest pH-related phenotype (Denison et al., 1998). Interestingly, and PalFp has N terminal and C terminal domains similar to those of mammalian arrestin (Herranz et al., 2005). PalFp strongly binds with the PalHp C-terminal cytoplamic region. At alkaline pH and in the presence of *PALH*, PalFp is phosphorylated to be activated and ubiquinated to be degraded. This post-translational regulation is partially dependent upon PalIp, but it is not dependent upon PalAp, PalBp, or PalCp, suggesting that PalHp and PalIp act upstream of PalFp and that PalAp, PalBp, and PalCp act downstream of PalFp (Herranz et al., 2005). Unlike the other Pal proteins, only PalCp does not have a homolog in *S. cerevisiae* and only has homologs in filamentous fungi. PalCp contains a Bro-1-like domain: the Bro-1 domain is also found in PalAp and the multi-vescicular body (MVB) pathway protein Bro1p/Vps31p. PalAp and Bro1p/Vps31p both interact with Vps32p/Snf7p, which is an MVB component [endosomal sorting complex required for transport (ESCRT)-III component]. Probably, PalCp is involved in endocytosis (Tilburn et al., 2005). PalAp is homologous to mammalian AIP1/Alix, which interacts with apoptosis-linked protein ALG-2, and PalAp and AIP1/Alix both interact with YPXL/I motif. One YPXL/I motif exists on both sides of the PacCp signaling protease box and are required for signaling protease cleavage. PalAp binds with $PacCp^{72}$ and may recruit protease PalBp to cleave PacCp at the signaling protease box (Vincent et al., 2003). Also, PalBp has a domain homologous to the catalytic domain of cysteine protease of the calpain family (Denison et al., 1995).

In 2001, the Mitchell laboratory showed that Rim20p interacts with the C terminus ~100 amino acids of Rim101p (Xu and Mitchell, 2001). Genome-wide yeast two-hybrid assay

shows that Vps32p/Snf7p interacts with Rim20p and Rim13p (Ito et al., 2000), and it also interacts with the MVB pathway protein Bro1p/Vps31p. ESCRT-I, -II, and -III complexes are all required for the activation of Rim101p (Xu et al., 2004), and Rim20p, Bro1p/Vps31p, and Vps32p/Snf7p co-localize under alkaline conditions (Boysen and Mitchell, 2006). These results indicate that the MVB-ESCRT pathway is associated with the activation of Rim101p.

4.14 Biological effects of PacCp/Rim101p

PacCp acts as a transcription factor in a pH signaling pathway. PacCp activates alkaline expressed genes and represses acidic expressed genes. Constitutive mutants *pacc*^{c} show alkaline mimic growth, constitutively activating alkaline expressed genes and constitutively repressing acidic expressed genes. Partial loss-of-function mutants *pacc*$^{+/-}$ and complete loss-of-function mutants *pacc*Δ show acidic mimic growth, with gene regulation effects similar to the wild type at acidic pH. The *pacc*^{c} mutants grow poorly at acidic pH, *pacc*$^{+/-}$ mutants grow poorly at alkaline pH, and *pacc*Δ mutants have slow growth rate and poor conidiation, and overproduce black pigment (Penalva and Arst, 2004).

The genes regulated by PacCp mainly include those encoding secreted enzymes, enzymes involved in the synthesis of exported metabolites, and permeases. The genes encoding the secreted enzymes include the acid phosphatase gene *PACA*, the alkaline phosphatase gene *PACD*, the alkaline protease gene *PRTA*, and the xylanase genes *XLNA* and *XLNB*.

The genes encoding enzymes involved in the synthesis of exported metabolites include the isopenicillin N synthase gene *IPNA*, and the genes encoding permeases include the γ-aminobutyrate (GABA) permease gene *GABA*. Of these genes, the acid phosphatase gene *PACA* and the xylanase gene *XLNB* are more highly expressed under acidic pH conditions or in *pal*Δ mutants, whereas the alkaline phosphatase gene *PACD*, the alkaline protease gene *PRTA*, and the xylanase gene *XLNA* are more highly expressed under alkaline pH conditions or in *pacc*^{c} mutants (MacCabe et al., 1998; Tilburn et al., 1995). The isopenicillin N synthase gene *IPNA* is also an alkaline-expressed gene, and its promoter has three PacCp binding sites that are bound by PacCp in gel shift assay. Mutation of the binding sites causes a decrease in the promoter activity under alkaline conditions (Espeso and Penalva, 1996). The acidic-expressed gene *GABA* has two PacCp binding sites on the promoter of *GABA,* which overlap with the binding site of the transcription activator IntAp. Consequently PacCp competes with IntAp to bind the promoter of *GABA* that is repressed under alkaline pH conditions. When the PacCp and IntAp binding sites on the *GABA* promoter were exchanged with the IntAp binding site on the promoter for the *AMDS* gene that is not pH regulated, the *GABA* promoter was no longer pH regulated, and the *AMDS* promoter became acid-expressed (Espeso and Arst, 2000). Furthermore, the expression of genes involved in biosynthesis and the uptake of siderophore is up-regulated at higher pH and is dependent upon PacCp (Eisendle et al., 2004).

Of interest to my research is the finding that certain *A. nidulans* mutation strains have been used to infect immuno-compromised mice and have then been observed in their

lungs. The *pacc*$^{+/-}$ and *palB*Δ strains were found to have less growth and less penetration of the lungs, while constitutive *pacc*^{c} strains have been found to increase the mortality of mice and to have more lung penetration. These results demonstrate that PacCp plays an important role in aspergillosis pneumonia pathogenesis (Bignell et al., 2005).

The *rim101*Δ mutant shows a number of defects, including inefficient sporulation, cold-sensitive growth at 17^{o}C, defective invasive growth, and sensitivity to high ion concentration (Lamb et al., 2001) (Li and Mitchell, 1997; Su and Mitchell, 1993a). Alkaline-expressed and Rim101- dependent genes include the Na^{+} pump gene *ENA1* and the siderophore-iron transporter gene *ARN4*. The genes targeted by Rim101p also include the transcription factor genes *NRG1* and *SMP1*, and Rim101p represses the expression of *NRG1* and *SMP1*. Chromatin IP shows that Rim101p binds to their promoters. The double mutants *rim101*Δ*nrg1*Δ and *rim101*Δ *smp1*Δ suppress the defects of *rim101*Δ on sporulation, salt stress, and invasive growth, and Nrg1p and Smp1p may act as repressors of the genes responsible for these phenotypes (Lamb and Mitchell, 2003). Furthermore, the *rim101*Δ mutant is sensitive to cell wall perturbation reagents, and the *rim101*Δ*slt2*Δ double mutant (Slt2p is the MAP kinase in the PKC pathway) is synthetic lethal, so that the Rim101p pathway and the PKC pathway may function in parallel in the regulation of cell wall construction (Castrejon et al., 2006).

In *C. albicans*, the 1,3-β-glucanosyltransferase genes, *PHR1*, and *PHR2* are pH-regulated genes that are dependent upon CaRim101p. *PHR1* is expressed in alkaline conditions, and *PHR2* is expressed in acidic conditions (Muhlschlegel and Fonzi, 1997; Ramon and

Fonzi, 2003; Saporito-Irwin et al., 1995). *PRA1*, which encodes a secreted glycoprotein and is orthologous to a surface antigen of certain *Aspergillus* species, is expressed under alkaline conditions and is also dependent upon CaRim101p (Sentandreu et al., 1998). *KER1*, which encodes a plasma membrane protein and is involved in cell aggregation, is also an alkaline-expressed gene and is dependent upon CaRim101p (Galan et al., 2004). The hyphal-specific GPI-CWP genes *HWP1* and *RBT1* are activated by CaRim101p, and the GPI-CWP genes *RBR1*, *RBR2*, and *RBR3* are repressed by CaRim101p and expressed under acidic conditions (Lotz et al., 2004). With respect to cell differentiation, *CaRIM101* is required for the induction of filamentous growth, and the activation of filamentous growth in *carim101*^{c} is dependent upon *EFG1* (El Barkani et al., 2000). From the point of view of pathogenicity, *carim101*Δ has been shown to be less virulent in a mouse model, less infectious to kidneys, and less infectious to endothelia cells (Davis et al., 2000a).

At pH 2.5, *W. dermatitidis* is induced to form isotropically enlarged forms that may become multi-cellular (sclerotic) by internal septation. Prior to their formation, yeast budding reproductive growth is repressed, but the isotropically enlarged, internally septated cells can still proliferate through slow fission. Such sclerotic morphotypes are found in the lesions of chromoblastomycosis, and microscopic observations and chemical analysis reveal that like those of *W. dermatitidis,* they have thicker, chitin-rich cell walls (Cooper, 1984; Harris, 1986; Roberts et al., 1979; Roberts et al., 1980; Szaniszlo, 2006; Szaniszlo et al., 1976). Calcium starvation, culture of the ts mutants *wdcdc1* (Mc2) and *wdcdc2* (Mc3) mutants at 37^{o}C, and culture of the *wdcdc42*^{G14V} dominant active mutant also induce the formation of sclerotic morphotypes (Cooper and Szaniszlo, 1993;

Karuppayil and Szaniszlo, 1997; Ye and Szaniszlo, 2000). To investigate the regulation of *WdPACC,* with respect to the growth of *W. dermatitidis* under different pH conditions, *WdPACC* was cloned, characterized and disrupted, and the resulting mutants studied.

4.2 MATERIALS AND METHODS

4.21 Culture conditions

Wangiella dermatitidis strain 8656 (ATCC34100) was the wild-type strain used in this study. *E.coli* strain used for the *W. dermatitidis* partial genomic library construction is DH10B. *E.coli* strain used for cloning is XL-1-blue. Routine culture of *W. dermatitidis* was on YPD agar (YPDA) and in YPD broth (YPDB) as described previously (Liu et al., 2004). For the induction of the filamentous growth, *W. dermatitidis* was grown on potato dextrose agar (PDA; Difco Scientific, Detriot, Mich.). For slide cultures**,** a thin square of PDA agar was placed on a slide and then inoculated on each side before being overlaid with a cover slip and incubated over water in a closed Petri dish. For the induction of sclerotic forms, *W. dermatitidis* was grown in modified Czapek Dox broth [MCDB; 3.5% Czapek Dox, 0.1% yeast extract, pH adjusted with HCl (Wang and Szaniszlo, 2000)].

4.22 Degenerate PCR

To design degenerate primers, PacCp family members from 10 filamentous fungi (*A. nidulans, A. parasiticus, A. oryzae, A. niger, P. chrysogenum, Sclerotinia sclerotiorum, Acremonium chrysogenum, Gibberella moniliformis, Gibberella fujikuroi, F. oxysporum*) were aligned to find the conserved regions. Degenerate primers were derived from their conserved zinc finger regions with sequences: WPF1, CAYGTIGGIMGIAARWSXACXAAYAA and WPR1: ISWRTCRTCIGCRTGXGTYTTXACRTG (Y=C+T, M=A+C, R=A+G, W=A+T, S=C+G, X=A+T+C+G). PCR amplifications were carried out with 2 μM of each primer, 0.5 mM dNTP, 0.5 μg genomic DNA and 0.5 U/μl Taq polymerase mixed in 1.5 mM Mg^{++}, 50 mM KCl, 10 mM Tris-HCl (pH 9) buffer. The PCR reaction conditions were as follows: 5 min at 94°C for premelting; 50 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C for extension; 7 min at 72°C for completion of the extension. The resulting PCR products were then cloned into pGEM-T easy vector (Promega, Madison, Wis.) and sequenced. The sequencing showed that plasmid pT-*WdPACC*204 contained a 204-bp-amplified sequence corresponding to the zinc finger regions of the PacCp proteins.

4.23 Construction and screening of a *W. dermatitidis* partial genomic library

Southern analysis of the genomic DNA was carried out by digestion with different restriction enzymes, which showed that *WdPACC* existed as a single gene. After a *W. dermatitidis* cosmid genomic library was screened with no success using the 204-bp

sequence as a probe (Feng et al., 2001), a partial genomic library was constructed for isolating the full-length gene. To construct the partial library, genomic DNA was first digested with *Xho*I and then after electrophoresis, a ~6 kb fragment was cut from the gel, purified by QIAquick gel extraction kit (Qiagen) and ligated into the *E. coli* vector pBSKS digested with *Xho*I and dephosphorylated with calf intestine alkaline phosphotase (CIAP, Promega). For the cloning itself, *E. coli* strain DH10B was cultured overnight in LB and shaken vigorously at 37°C for ~2 to 2.5 h until OD_{595} reached ~0.6. After the bacterial cells were collected by centrifugation and washed 2-3 times with ice-cold autoclaved 10% glycerol, they were finally resuspended in a small volume of ice-cold autoclaved 10% glycerol. Ligation products (1-2 µl), precipitated with ethanol, washed with 70% ethanol and dissolved in 5 mM Tris-Cl (pH8.0), were then mixed with 40 µl of the cells prior to transfer to a prechilled 0.2 cm gap cuvett (Bio-Rad). The gene pulser apparatus (Bio-Rad) was pulsed at 2.00 kV, 25 µF and 200 Ω. A pulse was with a time constant above 4 ms. After the pulse, LB (1 ml) was immediately added to the cuvett to quickly and gently resuspend the cells, and the resulting transformation culture incubated at 37°C with moderate shaking for 1 h, before plating on LB agar containing 100 µg/ml of ampicillin for overnight incubation at 37°C. A clone containing the *WdPACC* sequence was then successfully obtained by screening the partial genomic library with hybridization using the 204-bp sequence as a probe. Sequencing was finally carried out with the pBSKS vector and the gene specific primers using big dye chemistries on capillary-based ABI 3130 and ABI 3739 DNA analyzers (Applied Biosystems, Foster City, Calif.) (Core facility of Institute of Cellular and Molecular Biology, University of

Texas at Austin). The sequencing showed that 5859 bp *W. dermatitidis* sequence was inserted in the *Xho*I site of pBSKS. This plasmid was named pBSKS-WP5859.

4.24 RT-PCR

To locate the positions of the introns, RNA was extracted with hot acidic phenol from *W. dermatitidis* cells grown in YPDB for 24 h at 37°C and treated with RQ1 RNase-Free DNase (Promega). RT-PCR was carried out with primers WP17F: CTGCTCGAGATGTCTGAACTCGCCGAGACCAG, and WP18R: TCGCTCGAGGCGTTTGCGAGCCTCATAATC, using One-step RT-PCR kit (Qiagen). The amplification products were cloned into pGEM-T easy vector (Promega) and sequenced. Alignment of the cDNA sequence with the genomic DNA sequence showed that *WdPACC* had two introns.

4.25 *WdPACC* mutagenesis

To create the *WdPACC* mutation strains by a one-step replacement method, fragments containing the *hph* selection marker (Liu et al., 2004) flanked by *WdPACC* 5' and 3' sequences were constructed. For the loss of function mutant *wdpacc*$^{+/-}$, a 5' flanking sequence was amplified by PCR with primers WPF10: GGATCCCGAGTGTCAATTCAGCTGGA, and WPR10: *CTA*CTGGTTATGGTGATGTTGACCA (the *Bam*HI site that was introduced is underlined, and the stop codon is italicized). The products amplified were cloned into

the pGEM-T easy vector (Promega) to produce plamid pT-WP1 for sequencing. For *wdpacc*^{c}, a 5' flanking sequence was amplified by PCR with primers WPF11: GGATCCTCAGCTTCCCCAGATCATTC, and WPR11: *CTA*ACGGCGACGCTCTTCCTGATCATA (the *Bam*HI site that was introduced is underlined, and the stop codon is italicized). The amplified products were cloned to pGEM-T easy vector (Promega), to produce plasmid pT-WP2 for sequencing. After 0.5-kb *Bam*HI-*Sal*I fragments were released from both pT-WP1 and pT-WP2, they were cloned into the pBCKS vector (Stratagene), generating plasmids pBCKS-WP1 and pBCKS-WP2, respectively. After release of a 1.4-kb *Sal*I fragment containing the *hph* marker from pCB1636 (Fungal Genetics Stock Center, University of Kansas Medical Center), it was inserted into the *Sal*I site of pBCKS-WP1 and pBCKS-WP2 to also produce pBCKS-WP3 and pBCKS-WP4, respectively. The 3' flanking sequence was amplified by PCR with primers WPF12: GAACAGAGGCGTGGAGGTTA, and WPR12: GGTACCACGCGTACGACCCCTTACTA (introduced *Kpn*I site is underlined). The amplified products were cloned to pGEM-T easy vector (Promega) to produce plasmid pT-WP3 for sequencing. A 0.5-kb *Sal*I-*Kpn*I fragment was released from pT-WP3 and ligated into *Xho*I-*Kpn*I site of pBCKS-WP3 and pBCKS-WP4 to produce pBCKS-WP5 and pBCKS-WP6, repectively. After the 2.4-kb *Xba*I-*Kpn*I fragments were released from pBCKS-WP5 and pBCKS-WP6, they were gel purified and used to transform the *W. dermatitidis* wild-type strain. Transformants were selected on YPDA containing 50 μg/ml hygromycin. PCR with primers WPF16: CATCGACCCTGCTCTTGCA and WPR16: CGATGAGTCGCATGTTCTGAA were used to screen the transformants. The amplification products were analyzed by

electrophoresis in a 3% agarose gel together with a 2-log DNA marker (New England Biolabs, Ipswich, Mass.) as a size reference. Southern analysis of genomic DNA digested with *Sal*I and hybridized with the 204-bp *WdPACC* probe was used to confirm the mutants.

4.26 Analysis of *W. dermatitidis* growth by spot assays

The spot assays were carried out with log-phase yeast cells of *W. dermatitidis* cultured in YPDB at 25°C. After serial dilution, 10^5, 10^4, 10^3, and 10^2 cells in 5 µl were spotted with a micropipette onto agar media, and then incubated at 25°C and 37°C.

4.27 Northern analysis

For the Northern analysis, log-phase yeast cells of *W. dermatitidis* cultured in YPDB at 25°C were washed and inoculated to pH 7 and pH 3 MCDB at 10^6 cells/ml, and to pH 2.5 MCDB at 10^7 cells/ml. Cells were cultured with vigorous shaking at 25°C. Total RNA was isolated with the RNeasy kit (Qiagen). Briefly, cells were collected and ground thoroughly in liquid nitrogen with a mortar and pestle, followed by transfer to the guanidine isothiocyanate-containing buffer RLT, spinning through a QIAshredder, and applying to an RNeasy spin column. The concentration of RNA was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Del.). The quality of RNA was detected by electrophoresis of 2 µg RNA in a 1% agarose gel containing 2 M formaldehyde. For the Northern blotting itself, 10 µg RNA was run in a

1% agarose gel containing 2 M formaldehyde. A 0.24-9.5 kb RNA ladder (Invitrogen) was run simultaneously as a size marker. For the *WdPACC* probe, the 204-bp *WdPACC* fragment from pT-*WdPACC*204 plasmid was used. For *WdCHS5* probe, a fragment amplified by PCR using primers pATG and pIntron1 was applyed (Liu et al., 2004). *WdCHS1, WdCHS2, WdCHS3* and *WdCHS4* fragments for probes were amplified by PCR with their specific primers (Wang et al., 2002). After Northern hybridization, radioactive signals were detected by exposure to a phosphoimager screen which was then scanned with a Molecular Imager FX Pro Plus multiimager system (Bio-Rad, Hercules, Calif.).

4.3 RESULTS

4.31 Isolation and sequence characterization of *WdPACC*

To clone *WdPACC,* ten PacC family members of filamentous fungi were aligned to design degenerate primers from their conserved zinc finger DNA-binding domains. A PCR product amplified with the degenerate primers was then cloned, which sequencing showed that encoded a protein fragment corresponding to that of the PacCp zinc finger domain. Using this fragment as a probe, Southern analysis of genomic DNA digested with different restriction enzymes indicated that *WdPACC* existed as a single copy in the *W. dermatitidis* genome (Figure 4.2).

Because a *WdPACC* hybridization signal was not found in a screen of a *W. dermatitidis* cosmid genomic library, a *W. dermatitidis* subgenomic library was constructed for its cloning. Based on a Southern analysis result, the subgenomic library was constructed with ~6-kb fragments of *Xho*I-digested genomic DNA and a pBSKS vector. Over 10^4 colonies were screened before a transformant containing *WdPACC* was successfully identified.

Sequencing of the isolated plasmid revealed that the inserted *W. dermatitidis* sequence was 5,958 bp, and that the *WdPACC* ORF consisted of bp 2,855 to 4,912 (Figure 4.3, 4.4). Analysis of the upstream sequence using the MatInspector software (www.genomatix.de) identified three consensus PacCp binding sites at -796 bp, -949 bp and -1122 bp upstream of the ORF start codon. The binding site at -796 bp is on the sense strand, and the other two binding sites are on the anti-sense strand (Figure 4.4). Analysis of ORF showed that it encodes a putative protein of 646 aa (Figure 4.3, 4.4) with a theoretical isoelectric point (PI) of 6.15 and a molecular weight (MW) of 83.6 kDa. Protein structure predictions showed that WdPacCp contains three C_2H_2-type zinc fingers for DNA binding (residues 90 to 115, residues 126 to 150 and residues 156 to 178, based on Pfam database), has Asn- (12-27), Ala- (32-47) and Gln- (56-78) rich regions before the zinc finger motifs (Figure 4.4), and is an α-helix-rich protein.

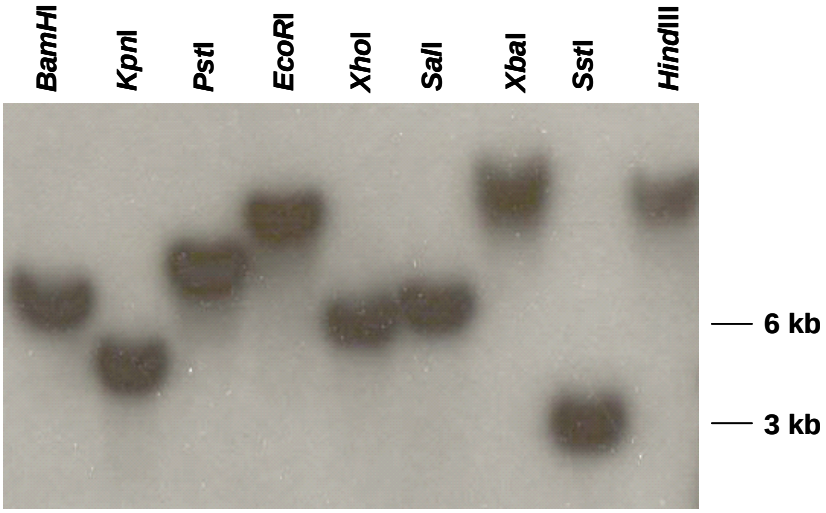


FIGURE 4.2. SOUTHERN ANALYSIS OF GENOMIC DNA OF *W. DERMATITIDIS* WILD TYPE HYBRIDIZED WITH THE *WDPACC* PROBE. The probe was the 204-bp *WdSTUA* PCR product. Genomic DNA was cut with the various enzymes indicated above each lane.

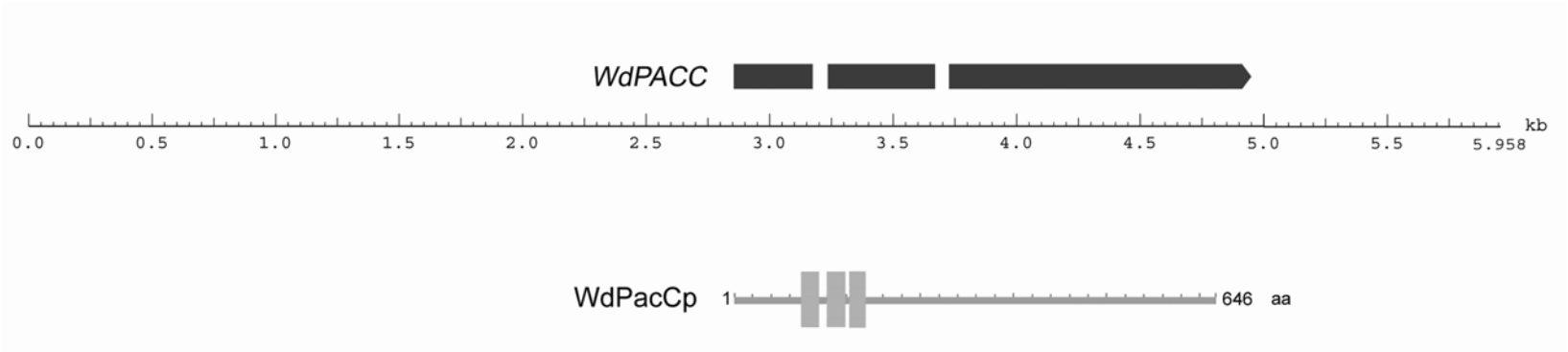
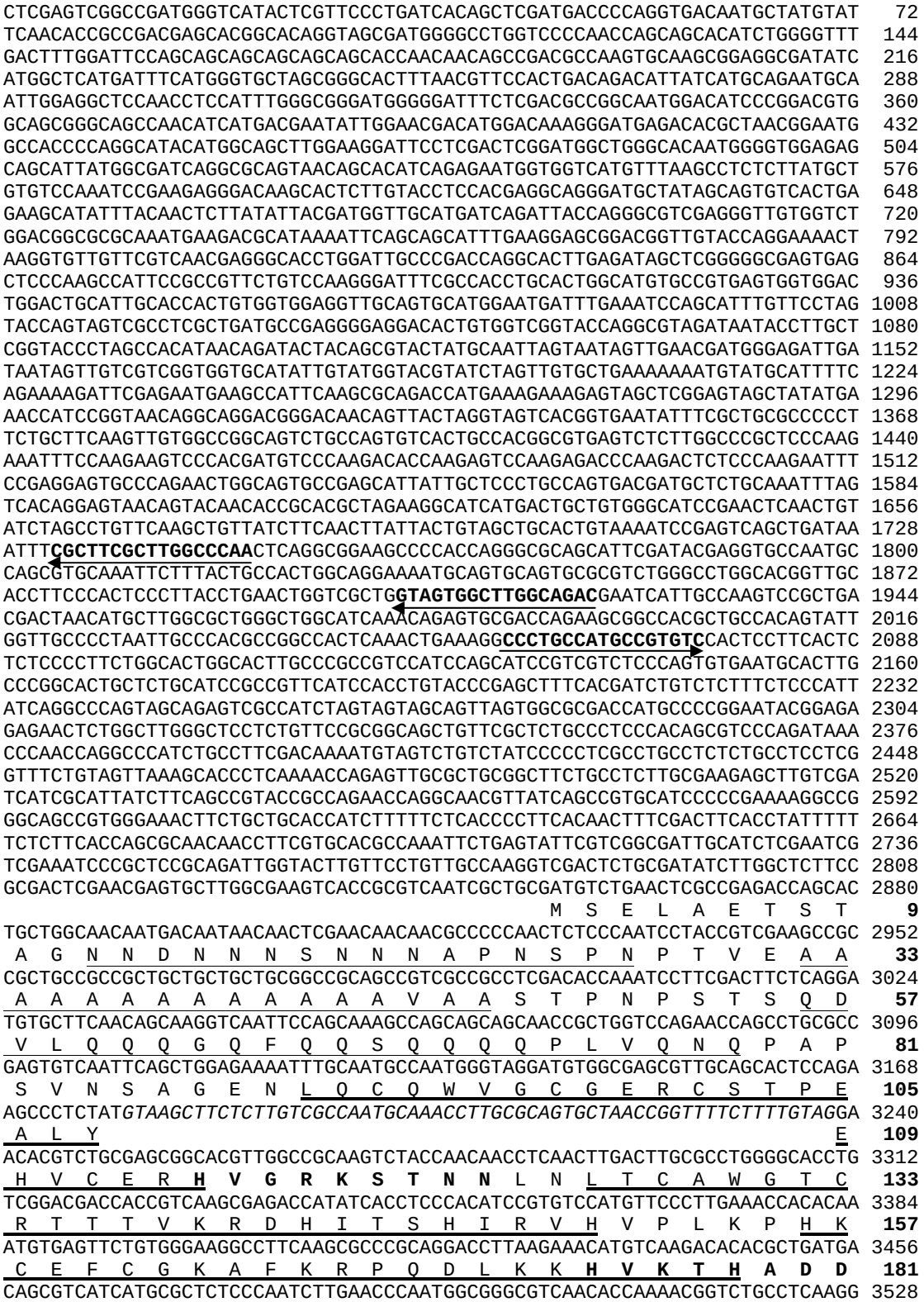


FIGURE 4.3. SCHEMATIC REPRESENTATION OF *WDPACC* AND WDPACCP. Diagram illustrating the cloned *WdPACC* locus (modified from the GENSCAN program; genes.mit.edu/GENSCAN.html). The two introns that interrupt the three exons of *WdPACC* are indicated by the two open areas in the arrow. The three C_2H_2-type zinc finger DNA-binding motifs are shown by the three small vertical bars.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TCAACACCGCCGACGAGCACGGCACAGGTAGCGATGGGGCCTGGTCCCCAACCAGCAGCACATCTGGGGTTT 144
GACTTTGGATTCCAGCAGCAGCAGCAGCACCAACAACAGCCGACGCCAAGTGCAAGCGGAGGCGATATC 216
ATGGCTCATGATTTCATGGGTGCTAGCGGGCACTTTAACGTTCCACTGACAGACATTATCATGCAGAATGCA 288
ATTGGAGGCTCCAACCTCCATTTGGGCGGGATGGGGGATTTCTCGACGCCGGCAATGGACATCCCGGACGTG 360
GCAGCGGGCAGCCAACATCATGACGAATATTGGAACGACATGGACAAAGGGATGAGACACGCTAACGGAATG 432
GCCACCCCAGGCATACATGGCAGCTTGGAAGGATTCCTCGACTCGGATGGCTGGGCACAATGGGGTGGAGAG 504
CAGCATTATGGCGATCAGGCGCAGTAACAGCACATCAGAGAATGGTGGTCATGTTTAAGCCTCTCTTATGCT 576
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GAGAACTCTGGCTTGGGCTCCTCTGTTCCGCGGCAGCTGTTCGCTCTGCCCTCCCACAGCGTCCCAGATAAA 2376
CCCAACCAGGCCCATCTGCCTTCGACAAAATGTAGTCTGTCTATCCCCCTCGCCTGCCTCTCTGCCTCCTCG 2448
GTTTCTGTAGTTAAAGCACCCTCAAAACCAGAGTTGCGCTGCGGCTTCTGCCTCTTGCGAAGAGCTTGTCGA 2520
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TCTCTTCACCAGCGCAACAACCTTCGTGCACGCCAAATTCTGAGTATTCGTCGGCGATTGCATCTCGAATCG 2736
TCGAAATCCCGCTCCGCAGATTGGTACTTGTTCCTGTTGCCAAGGTCGACTCTGCGATATCTTGGCTCTTCC 2808
GCGACTCGAACGAGTGCTTGGCGAAGTCACCGCGTCAATCGCTGCGATGTCTGAACTCGCCGAGACCAGCAC 2880
M S E L A E T S T 9
TGCTGGCAACAATGACAATAACAACTCGAACAACAACGCCCCCAACTCTCCCAATCCTACCGTCGAAGCCGC 2952
A G N N D N N N S N N N A P N S P N P T V E A A 33
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TGTGCTTCAACAGCAAGGTCAATTCCAGCAAAGCCAGCAGCAGCAACCGCTGGTCCAGAACCAGCCTGCGCC 3096
V L Q Q Q G Q F Q Q S Q Q Q Q P L V Q N Q P A P 81
GAGTGTCAATTCAGCTGGAGAAAATTTGCAATGCCAATGGGTAGGATGTGGCGAGCGTTGCAGCACTCCAGA 3168
S V N S A G E N L Q C Q W V G C G E R C S T P E 105
AGCCCTCTATGTAAGCTTCTCTTGTCGCCAATGCAAACCTTGCGCAGTGCTAACCGGTTTTCTTTTGTAGGA 3240
A L Y E 109
ACACGTCTGCGAGCGGCACGTTGGCCGCAAGTCTACCAACAACCTCAACTTGACTTGCGCCTGGGGCACCTG 3312
H V C E R H V G R K S T N N L N L T C A W G T C 133
TCGGACGACCACCGTCAAGCGAGACCATATCACCTCCCACATCCGTGTCCATGTTCCCTTGAAACCACACAA 3384
R T T T V K R D H I T S H I R V H V P L K P H K 157
ATGTGAGTTCTGTGGGAAGGCCTTCAAGCGCCCGCAGGACCTTAAGAAACATGTCAAGACACACGCTGATGA 3456
C E F C G K A F K R P Q D L K K H V K T H A D D 181
CAGCGTCATCATGCGCTCTCCCAATCTTGAACCCAATGGCGGGCGTCAACACCAAAACGGTCTGCCTCAAGG 3528

S V I M R S P N L E P N G G R Q H Q N G L P Q G **205**
TGGCGACTATGCCTCATACTATGGCCATGGTCTGACAGGACAAGTCGCTCAAAGCTACTACGCTCCTGCGAT 3600
G D Y A S Y Y G H G L T G Q V A Q S Y Y A P A M **229**
GCCTGGGCCTCAAGACTATACTGGTCAACATCACCATAACCAGTATTACCAGCCGCATCCACCTGTTTCC*GT* 3672
P G P Q D Y T G Q H H H N Q Y Y Q P H P P V S **252**
*AAGTGCCATGGCCCAATCTGACCAGCACAGCTTGTATACTTACACGTACCCGCAG*TCAGGGCATCCCGGTTA 3744
S G H P G Y **258**
CGGCCCGGTGACTTACTACAGCACCGCCAGTACCACCAGTCCTTATGATTATGAGGCTCGCAAACGCGGGTA 3816
G P V T Y Y S T A S T T S P Y D Y E A R K R G Y **282**
TGATGCTCTTGATCATTTCTTTGGCCAGGTCAAACGCAGAGAGTTGGACCCGATCAACTTCCACAACATCAG 3888
D A L D H F F G Q V K R R E L D P I N F H N I S **306**
CCGCCGTCTTTTTGAGCTCCAAGGTCTTCAGCTTCCCCAGATCATTCCCTCCAGCGTCCCGGCGCCCGCTTA 3960
R R L F E L Q G L Q L P Q I I P S S V P A P A Y **330**
TCAGCCGGTTTCTGTGGGTGGAGCCTACGAAGCGGCTGATCCTATCCAAGCCTACAGCTTGCCTCCCATGGG 4032
Q P V S V G G A Y E A A D P I Q A Y S L P P M G **354**
AAATGCAAAGACTCGCGAAGATTTGACCTCGATCGACCAGATCCTGGAGCAGATGCAAGCCACCATTTATGA 4104
N A K T R E D L T S I D Q I L E Q M Q A T I Y E **378**
GAACGATACTCACCTGGGTTCGGCGAATGTTGGCCAGAGTGGTTCAGGCTACGTTGCCTACCGCACAAGCAA 4176
N D T H L G S A N V G Q S G S G Y V A Y R T S N **402**
CAGCCCGCCCAGCACAAGCCAGCAGCCGTCCACGGCCGCGCAGACGAATCCGACTGCCTTGCTCCAAAATCA 4248
S P P S T S Q Q P S T A A Q T N P T A L L Q N Q **426**
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T Q S N V A S N T D A S T P G L T P P S S A Q S **450**
CTACACCTCTGGTCACAGCCCGTTACAAGGCCATACTGCCCCCTCCGGTAACGGCCTGTATCCCAATCTGCC 4392
Y T S G H S P L Q G H T A P S G N G L Y P N L P **474**
ATCCAGTGCCAGTGACATGGCTTATGCTGCGGCCAACGCCGCCACTCTCAGTGGAATTTATGATCAGGAAGA 4464
S S A S D M A Y A A A N A A T L S G I Y D Q E E **498**
GCGTCGCCGTTATAGCGGTGGCATGCTTCAACGAGCCGCTCCTGCCAAGGTCAAGGATGAAATGGACATCAC 4536
R R R Y S G G M L Q R A A P A K V K D E M D I T **522**
TAGCGACGGGTCTGTTACTCCACCTGCCTCGGCACTGAACCAGCAAAACAAGGACAAGAAGTCGGCGTCTCC 4608
S D G S V T P P A S A L N Q Q N K D K K S A S P **546**
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Q E N V I D P A L A A E A A S T P K S D P R D A **570**
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D Q E Q V W V Q N M R L I E W M R E F I K K K L **594**
CCAGAGCGGCGACTATGAGGAGGAAAACGGTCACGGAGGTTCTCCTCACCATGGCGGTCAGCTTGACCATGA 4824
Q S G D Y E E E N G H G G S P H H G G Q L D H D **618**
CATGAGCGGGACCAGTGACCAAAAACATGACAAGTCAGAAGAGGAGCAACTCTATCCTGTCCTGCGACATGT 4896
M S G T S D Q K H D K S E E E Q L Y P V L R H V **642**
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E E S A * **646**
GTAGACTGTCCTTGCTCCTACGAATACCTGGTGTCGATTTCGAATTTGGTTTGCTTCTCCGAACGCCGGTCA 5040
GAGGCGTCATATCAAATTAAAAAAAAATCTGTGGCGCTCCAACTGGAACAGCATGAAAAGGGGTTTCATCAT 5112
TACCGCCTGACTTAATGGGAGAGGATCAAAGGTGTTTTAATCCACGGAGTGGGATGGGCCGGGCTGGCGCAG 5184
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TGAGTAGCAGAAGCAAAGCTCATCACGCAGAACCAGCCTCAACTTGAGCTCGAG 5958

FIGURE 4.4. *WDPACC* NUCLEOTIDE SEQUENCE AND ITS DERIVED AMINO ACID SEQUENCE. The amino acids for the design of degenerate primers are shown in bold. The three zinc finger motifs (based on Pfam database) are underlined with thick lines. The Asn-, Ala- and Gln- rich regions before the zinc finger motifs are underlined with thin lines. The intron sequences are displayed by italic letters. In the promoter region, the PacCp binding sites including the core and flanking sequences are shown in bold, with orientations are indicated by arrows. The *WdPACC* loss-of-function mutation and the *WdPACC* constitutive mutation were designed to truncate after Q243 and R501, respectively, which are shaded.

A

```
AoPacCp         ---------------MSEPQDTTSPSTAAAPIAASTSHEQPQTQ-SPPQVSATTTSSVT 43
AnPacCp         ---------------MLG----AMAEEAVAPVAVPTTQEQPTSQPAAAQVTTVTSPSVT 40
PcPacCp         ------------------------MTENHTPSTTQPTLPAPVAEAAPIQANPAPSASVT 35
TrPacCp         ----------MQSASSLQQQQPQQQQQQQPQQQGQQQGQQAQQQQGQQQQPASQTVQSVP 50
WdPacCp         ---------MSELAETSTAGNNDNNNSNNNAPNSPNPTVEAAAAAAAAAAAAAAVAASTPN 51
FoPacCp         MS-----------------PPAPEQKP--------QLQQQQQQGSSSG--------DSSS 27
NcPacCp         MSSTPAQENGTVNGANAAPAPAPAQTTPTPAPATAATPTTAPAASANGTAANAMKPEASS 60
                                                                        :

AoPacCp         ATA----AAATAAVA-SPPVNG-----------AARPTEELSCLWQGCSEKCPTPESLYE 87
AnPacCp         ATA----AAATAAVA-SPQANGNAASPVAPASSTSRPAEELTCMWQGCSEKLPTPESLYE 95
PcPacCp         ATA----AAATAAVNNAPSMNG-------------AGEQLPCQWVGCTEKSPTAESLYE 77
TrPacCp         VQANLQHAAAAAAAVAPPGVQQVAPN-------SPSAPGSLECQWQGCQELCPTPEALYE 103
WdPacCp         PSTSQDVLQQQGQFQQSQQQQPLVQN--QPAPSVNSAGENLQCQWVGCGERCSTPEALYE 109
FoPacCp         GSAN-DSKSVTPAPSATSNTSQSSTAP------STSSDDNLICRWNACNQKFPAPEALYE 80
NcPacCp         NSSNSASNGTTPAPSTTPTTASNSSAP------AAAQDESLVCRWAECNERFTSAEVLYE 114
                  :            .                     .* * *  * :  .:.* ***

AoPacCp         HVCERHVGRKSTNNLNLTCQWGSCRTTTVKRDHITSHIRVHVPLKPHKCDFCGKAFKRPQ 147
AnPacCp         HVCERHVGRKSTNNLNLTCQWGSCRTTTVKRDHITSHIRVHVPLKPHKCDFCGKAFKRPQ 155
PcPacCp         HVCERHVGRKSTNNLNLTCQWGTCNTTTVKRDHITSHIRVHVPLKPHKCDFCGKAFKRPQ 137
TrPacCp         HVCERHVGRKSTNNLNLTCGWSNCRTTTVKRDHITSHIRVHVPLKPHKCDFCGKAFKRPQ 163
WdPacCp         HVCERHVGRKSTNNLNLTCAWGTCRTTTVKRDHITSHIRVHVPLKPHKCEFCGKAFKRPQ 169
FoPacCp         HICERHVGRKSTNNLNLTCQWNSCRTTTVKRDHITSHIRVHVPLKPHKCEFCGKSFKRPQ 140
NcPacCp         HICEKHVGRKSTNNLNLTCQWNSCRTTTVKRDHITSHVRVHVPLKPHKCDFCGKCFKRPQ 174
                *:**:************** *..*.************:***********:****.*****

AoPacCp         DLKKHVKTHADDSVLVRSPEP---GSRNPD--IMFGGNPAKGYAT------ATHYFEPAL 196
AnPacCp         DLKKHVKTHADDSVLVRSPEP---GSRNPD--MMFGGN-FKGYA-------AAHYFEPAL 202
PcPacCp         DLKKHVKTHADDSE-IRSPEP---GMKHPD--MMFPQN-PRGSPA------ATHYFESPI 184
TrPacCp         DLKKHVKTHADDSVLMRSPEP---GAGQRQ--QPPAGMFGVGLG---------PDGKRSL 209
WdPacCp         DLKKHVKTHADDSVIMRSPNLEPNGGRQHQNGLPQGGDYASYYGHGLTGQVAQSYYAPAM 229
FoPacCp         DLKKHVKTHADDSVLVRPS-QDPQGGLN--YRPQPP--KGPSSYYD-----HTGQMR-TN 189
NcPacCp         DLKKHVKTHADDSVLVGRSPQDQNGGMNGAYRAQAPVHKAPSGFYD-----HNGHMRGTN 229
                ************ ::  .     *  :                          .

AoPacCp         NPVPSQGYAHGAPQYYQSHHPPQPANP-SYGNVYYALNHGH-----EAGHASYESKKRGY 250
AnPacCp         NPVPSQGYAHGPPQYYQAHHAPQPSNP-SYGNVYYALNTGP-----EPHQASYESKKRGY 256
PcPacCp         NGINGQYSHAPPPQYYQPHPPPQAPNPHSYGNLYYALSQGQ-----EGGHP-YD-RKRGY 237
TrPacCp         GPVP-QAYGHPPPQYYQPQHPQQQPNP-SYGNVYYAVGHD------AAHQASYESKKRGY 261
WdPacCp         PGPQDYTGQHHHNQYYQPHPPVSSGHP-GYGPVTYYSTAS------TTSPYDYEARKRGY 282
FoPacCp         AAAFAHQAGHPSGGYYAPQ-PSTN---YGLYFNQPPINNARTEHLGYSAAAGGYDRKRTY 245
NcPacCp         QVPFGQPHQNGQASYYHAQYPASQPYHAPMYYPAQTMGGQRNDFTGHQAAP-FDARKRQF 288
                              ** .: .                                  :** :

AoPacCp         DALNEFFGDLKRRQFDPNSYAAVGQRLLGLQS-LSLPILSGGPLPEYQPMPAPVAVGG-- 307
AnPacCp         DALNEFFGDLKRRQFDPNSYAAVGQRLLSLQN-LSLPVLTAAPLPEYQAMPAPVAVAS-- 313
PcPacCp         DALNEFFGDLKRRQFDPNSYAAVGQRLLGLQA-LQLPFLSG-PAPEYQQMPAPVAVGG-- 293
TrPacCp         DALNEFFGDLKRRQFDPTSYAAVGQRLLNLHG-LPLPLTHAGAVPEYQPMPAMVGVGGGH 320
WdPacCp         DALDHFFGQVKRRELDPINFHNISRRLFELQG-LQLPQIIPSSVPA--PAYQPVSVGG-- 337
FoPacCp         DMVDDFFGSAKRRQIDPSSYAQIGRSLMPLHGNLSVPNGPMTATEQYMPQPAPAPVHAG- 304
NcPacCp         DDLNDFFGSVKRRQINPTSYESVGRALMPLHAPLGLHSGGLAT--EYMAQP---PHTLG- 342
                * ::.***. ***:::* .:  :.: *: *:  * :      .
                          I
AoPacCp         GGYSPGGHPP-APAYHLPPMSNVRTKNDLINIDQFLQQMQDTIYENDDNVAAAGVAQPGA 366
AnPacCp         GPYG-GGPHP-APAYHLPPMSNVRTKNDLINIDQFLQQMQDTIYENDDNVAAAGVAQPGA 371
PcPacCp         GGGGYGGGAPQPPGYHLPPMSNVRTKNDLINIDQFLEQMQNTIYESDENVAAAGVAQPGA 353
TrPacCp         GGYQSAGPIP-TQSYHLPPMGNLRTKADLMNIDQFLEQMQSTVYESDENVAAAGVAQPGA 379
WdPacCp         ---AYEAADP-IQAYSLPPMGNAKTREDLTSIDQILEQMQATIYENDTHLGSANVGQSGS 393
FoPacCp         ---PTPSKTPSAQQYYLP-MPSARTQKDLIHIDTILGQMQDTIYENANHATAGVHIHHAE 360
NcPacCp         ---MASAHHPLTQHYYLPPMPNLRTKEDLQQMDHFLEQMQATVYENT-----AVDMRHHS 394
                      .  *    * ** * . :*: **  :* :* *** *:**.     .   :
                                 II
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```
AoPacCp         H---YVHGGMSYRTTHSPPSQLPPSHATATTSAGPIMAN---------------------- 402
AnPacCp         H---YIHNGISYRTTHSPPTQLPSAHATTQTTAGPIISN---------------------- 407
PcPacCp         H---YVHGGMNHRTTHSPPT--HSRQATLLQLPSAPMAA---------------------- 387
TrPacCp         H---YVQGPLSYRTTNSPPTHHQSHHQHPHATATAAATTTAATTASMMSTSAAATPASSI 436
WdPacCp         G---YVA----YRTSNSPPSTSQQPSTAAQTNPTALLQN------------------QTQ 428
FoPacCp         NGFNGYRNTPSPPTSHRSPTGMHVGADGYQPVSAASMAS-------------------P 400
NcPacCp         ------------PTYATRPS--------IDPYHGASLAS-------------------P 414
                             *    *:           .

AoPacCp         -PATHSPTG-TPALTPPSSAQSYTSGRSPISLPSTSRVSPPHHEGGSSMYPRLPSATMSD 460
AnPacCp         -TSAHSPSSSTPALTPPSSAQSYTSGRSPISLPSAHRVSPP-HESGSSMYPRLPSAT--D 463
PcPacCp         -ATAHSPSVGTPALTPPSSAQSYTSNRSPISLHSS-RVSPPHEEAAPGMYPRLPAAICAD 445
TrPacCp         SAASRSPHASTPALTPPSSAQSYTSGRSPISLASSHGMSPSHHPSTAGMYPTLPATTGQD 496
WdPacCp         SNVASNTDASTPGLTPPSSAQSYTSGHSPLQGHTA--------PSGNGLYPNLPSSASDM 480
FoPacCp         LTAIS--STGTPAVTASSS-MSYTSGHSPSPSSSAMSPQSRHGSTASVMYPTLPTSLPAV 457
NcPacCp         LSATSPHSAGTPAVTPTPSNMSYTSGHSPSTSSTSLSPTSRHSSTPSVSYPTLPS----- 469
                          **.:*...*  ****.:**    ::              ** **:
                                    III
AoPacCp         SMAA-GYP-TTSSAAPPSTLGGIFDHDDRRRYTGGTLQRARPEERHLPEP-MDLSHDNKD 517
AnPacCp         GMTS-GYT-AASSAAPPSTLGGIFDNDERRRYTGGTLQRARPASRAASES-MDLSSDDKE 520
PcPacCp         SMTA-GYP-TASGAAPPSTLSGAYDHDDRRRYTGGTLQRARPAERAATEDRMDISQDSKH 503
TrPacCp         SLSSSGYPTTVSSAAPPSTLSSVFD-DDRRRYTGGMLQRSRPDIDLSTPS-IKRDDADAT 554
WdPacCp         AY----------AAANAATLSGIYDQEERRRYSGGMLQRAAP---AKVKDEMDITSD--- 524
FoPacCp         SQGFG--------HSATTTLGPSFDGSERRRYSGGMLQRARAGPLPLPHEDTSGASTPKA 509
NcPacCp         RPGLP--------YPSTSGLGSNFTHNERR-LSGGVLQSARR-----AADEADRAPTPKA 515
                             . .: *.  :  .:**  :** ** :           .
                                       IV
AoPacCp         DGERTPPAKPRQAPSSPGRISASLIDPALS-GSANEAE---------TMRTAQAATEVAE 567
AnPacCp         SGERTP-----------KQISASLIDPALHSGSPGEDD---------VTRTAKAATEVAE 560
PcPacCp         DGERTP---------KAMHISASLIDPALSGTSSDPEQE-------SAKRTAATATEVAE 547
TrPacCp         AGKDEP-----------KLSSSVIDPALSRASVDMDEDAAPHRSPSSTPTPTATTAAAG 602
WdPacCp         -GSVTP-----------PASALNQQNKDKKSASPQEN----VIDPALAAEAASTPKSD 566
FoPacCp         SESALS-----------------------------------------VGSPSSESDVSD 527
NcPacCp         SEQAT------------------------------------------VSSPSEDSETGD 532
                  .                  :                          :    :  .

AoPacCp         RSDVQ------WVEKVRLIEYLRNYIASRLERGEYDGDS-------------------G 601
AnPacCp         RSDVQSE----WVEKVRLIEYLRNYIANRLERGEFSDDSEQEQDQEQEQDQEQEQDQEQG 616
PcPacCp         R-DVNVA----WVEKVRLLENLRRLVSGLLEAGSLTPEY-------------------G 582
TrPacCp         PDDRQPAGEQQWVENVRLLQRLRDYVLERLNNGDYVDEDKQDDSDKEEDAKSDKASPESA 662
WdPacCp         PRDADQE--QVWVQNMRLIEWMREFIKKKLQSGDYEEEN-------------------G 604
FoPacCp         ATREREEQYDRWLENMRVIETLREYVRGRLERKEFVDDNES------------------- 568
NcPacCp         VNGP--ETYDDWLQHMRVIEYLRQGIRARLERQDFDEDDTSR--------------IDPM 576
                          *::::*::: :*  :   *:  .   :
                                    V
AoPacCp         MTRESRT----------------------------PEAGPDGHMEGVETEPVS-HPAKC 631
AnPacCp         QDRVSRS----------------------------PVSKADVDMEGVERDSLPRSPRTV 647
PcPacCp         VQTSSAS----------------------------PTPGLD-AMEGVETASVR--AASE 610
TrPacCp         SASDAATGAGEYPSIKMHGMEAIAAAAVAHEEGRDDQMPRDDEDQENPSTPTARSTTTML 722
WdPacCp         HGGSPHHG---------------------------GQLDHDMSG------------- 621
FoPacCp         -PRSSHS----------------------------DAMDVDPKSPQ--AP------- 587
NcPacCp         VLESSDR----------------------------NQQQRNQQQQQQKSPN------ 599
                    .                                  :   .

AoPacCp         ESPVKPEAGGDTVMYPTLRGVDED-----GDSKMPN 662
AnPacCp         PIKTDGESAEDSVMYPTLRGLDED-----GDSKMPS 678
PcPacCp         QAREEPKSESEGVFYPTLRGVDEDED---GDSKMPE 643
TrPacCp         DAEEEAKQAGENLYPVLKMAVDDDGADTDGDEKMGQ 758
WdPacCp         TSDQKHDKSEEEQLYPVLRHVEESA----------- 646
FoPacCp         -PRELGTPREGSSLYPILRMPGA------------- 609
NcPacCp         EPTAAGPSAPEKPLYPVLPRIN-------------- 621
                                     :
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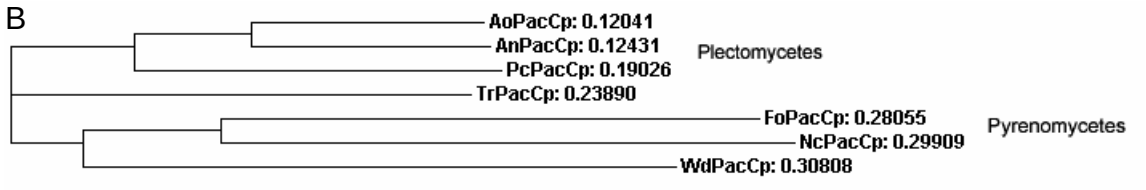


FIGURE 4.5. MULTIPLE ALIGNMENT AND PHYLOGRAM ANALYSIS OF WDPACCP WITH ITS CLOSELY RELATED ORTHOLOGS BY THE CLUSTALW PROGRAM. (A) *A. nidulans* AnPacCp accession #: EAA63426, *A. oryzae* AoPacCp accession #: BAE57899, *Penicillium chrysogenum* PcPacCp accession #: AAC36492, *Trichophyton rubrum* TrPacCp accession #: AAK35072, *F. oxysporum* FoPAcCp accession #: AAM95700, *N. spora* NcPacCp accession #: Q7RVQ. Stars indicate the identical amino acids, two dots show the similar amino acids, and single dots present the less conserved amino acids. The three zinc finger motifs are underlined with thick lines, and the other five homologous domains are illustrated by thin lines. (B) Phylogram exhibits the evolutionary relationship among these protein sequences.

Table 4.1. Homology of WdPacCp to various PacCp/Rim101p family members by BLASTP search

Organism	WdPacCp homologs	Identity (%)	Positive (%)	Homologous region in WdPacCp (aa)	Score (bits)
Aspergillus nidulans	AnPacCp	42	56	22-599	374
Aspergillus oryzae	AoPacCp	44	57	81-644	361
Penicillium chrysogenum	PcPacCp	40	52	18-644	331
Trichophyton rubrum	TrPacCp	42	54	78-640	349
Fusarium oxysporum	FoPacCp	39	53	76-640	280
Neurospora crassa	NcPacCp	38	53	88-603	239
Yarrowia lipolytica	YlRim101p	40	53	76-313	138
Ashbya gossypii	AgRim101p	46	59	38-187	132
Candida albicans	CaRim101p	58	67	74-181	123
Saccharomyces cerevisiae	ScRim101p	59	68	90-186	126
Ustilago maydis	UmRim101p	42	57	32-180	120

Table 4.2. Homology of the nucleotide sequence of *WdPACC* by BLASTN search

Organism	*WdPACC* homologs	Identity (%)	Homologous region in *WdPACC* (nt)	Length (nt)	Score (bits)
Trichophyton rubrum	*TrPACC*	83	3235-3450	216	151
Aspergillus oryzae	*AoPACC*	82	3236-3456	221	129
Fusarium oxysporum	*FoPACC*	82	3248-3442	195	109
Aspergillus nidulans	*AnPACC*	79	3239-3457	219	85
Penicillium chrysogenum	*PcPACC*	83	3326-3444	119	77
Ashbya gossypii	*AgRIM101*	90	3329-3378	50	59
Neurospora crassa	*NcPACC*	82	3354-3444	91	54

Comparison of the cDNA sequence obtained by RT-PCR and the genomic DNA sequence revealed the presence of two introns of 60 bp and 57 bp (bp 3,179 to 3,238 and bp 3,671 to 3,727) (Figure 4.4). The first intron is between Y108 and E109 in the first zinc finger, whereas the second intron, which does not introduce stop codons or frame shifts, is located between S252 and S253, and is about 30 residues upstream of the conserved processing protease cleavage site. Both introns begin with GT at their 5' ends and end with AG at their 3' ends and therefore conform to the "GT-AG" rule.

BLATP (www.ncbi.nlm.nih.gov/BLAST) analysis indicated that WdPacCp was most similar to the PacCp proteins of the other Ascomycota conidiogenous fungi. Among these, WdPacCp had a higher homology score with AnPacCp, AoPacCp, PcPacCp, and TrPacCp than with FoPacCp and NcPacCp. In addition, among ascomycota yeast fungi (*Saccharomycetes*), YlRim101p and AgRim101p were closer to WdPacCp than were ScRim101p and CaRim101p (Table 4.1). Multiple alignment of WdPacCp, AnPacCp, AoPacCp, PcPacCp, TrPacCp, FoPacCp and NcPacCp showed that the most conservation of the protein was in zinc finger 2 and 3, which have been found to make direct interactions with DNA and are involved in nuclear localization (Espeso et al., 1997; Fernandez-Martinez et al., 2003; Mingot et al., 2001; Tilburn et al., 1995). In addition, five other regions are relatively conserved: a signaling protease box (484-510) is in region IV; a predicted bipartite nuclear localization signal (279-296) is at region I; Regions II, III and V contain important interaction residues (Mingot et al., 1999) (Figure 4.5A). Phylogram analysis showed that WdPacCp was in a branch by itself among the conidiogenous filamentous fungal PacCp orthologs analyzed, but nonetheless appeared

more related to those of Pyrenomycetes than with those of the Plectomycetes (Figure 4.5B). At the nucleotide level, *WdPACC* homology was only found at the region coding for the DNA binding domain, with the highest homology being with that of *TrPACC, AoPACC* and *FoPACC*, which had more than 80% identical nucleotides (Table 4.2).

4.32 *WdPACC* is more expressed at neutral-alkaline pH than at acidic pH

Northern analysis of RNA from wild-type cells cultured for 24 h in pH 7 and pH 3 MCDB at 25°C showed that *WdPACC* was more highly expressed at pH 7 than at pH 3 (Figure 4.6). Thus, *WdPACC* RNA expression was pH regulated.

4.33 *WdPACC* constitutive and loss-of-function mutations have varied effects

Because no *wdpacc*Δ null mutants were obtained after screening numerous transformants, suggesting that *WdPACC* deletion may be a lethal event in *W. dermatitidis*, the function of *WdPACC* was studied using a constitutive (*wdpacc*^{c}) and a loss-of-function (*wdpacc*$^{+/-}$) mutant forms of *WdPACC* constructed according to those of *A. nidulans* (Tilburn et al., 1995), and derived by a one-step replacement method (Figure 4.7, 4.8). In *wdpacc*^{c}, WdPacCp is truncated after residue R501 in the conserved signaling protease box. In *wdpacc*$^{+/-}$, WdPacCp is truncated after residue Q243 at about 40 residues upstream of the conserved processing protease cleavage site.

Hygromycin resistant transformants were first screened by PCR diagnosis of their genomic DNA with specific primers located in the deleted region. The wild type showed a 100-bp band, while the mutants did not (data not shown). Among the transformants for *wdpacc*^{c}, approximately 1 out of 6 transformants had the deletion. These identified transformants did not have abnormal phenotypes on the YPDA-hygromycin medium compared to the other transformants. To identify *wdpacc*$^{+/-}$ strains, 39 transformants were screened by the PCR, and three of them had the deletion. All three identified transformants produced smaller colonies.

The identified *wdpacc*^{c} and *wdpacc*$^{+/-}$ strains were further confirmed by Southern hybridization of *Sal*I digested genomic DNA, using the 204-bp *WdPACC* fragment in the DNA binding domain as a probe. The wild type showed a 7-kb band, all the identified *wdpacc*^{c} strains showed a 1.7-kb band and all the identified *wdpacc*$^{+/-}$ strains showed a 0.9-kb band, which are consistent with the design (Figure 4.7B, 4.8B).

Because preliminary studies of all *wdpacc*$^{+/-}$ strains and all *wdpacc*^{c} strains showed identical phenotypes, the detailed comparison with the wild type described below was with *wdpacc*$^{+/-}$*-1* and *wdpacc*^{c}*-1***.**

4.34 The *wdpacc*$^{+/-}$*-1* mutant grows slower than the wild type or *wdpacc*^{c}*-1* under the normal conditions

Because the *wdpacc*$^{+/-}$*-1* mutant produced smaller colonies on YPDA containing hygromycin, this loss-of-function mutation strain was also tested when hygromycin was not present (Figure 4.9). The results showed that the growth of *wdpacc*$^{+/-}$*-1* was obviously retarded, whereas the amount of growth of *wdpacc*^{c}*-1* was similar to that of the wild type, although somewhat darker.

4.35 The *wdpacc*$^{+/-}$*-1* mutant is more sensitive to Na^+ stress than the wild type or *wdpacc*^{c}*-1* mutant

Because the PacCp proteins of some filamentous fungi are involved in the regulation of Na^+ stress, the effects of Na^+ stress in the *WdPACC* mutation strains spotted on YPDA and YPDA supplemented with 0.4 M NaCl and cultured at 25°C and 37°C were evaluated. The growth of the wild type was reduced on YPDA supplemented with 0.4 M NaCl at both temperatures. Although the growth pattern of *wdpacc*^{c}*-1* was similar to that of the wild type on both media at both temperatures, the *wdpacc*$^{+/-}$*-1* mutant grew more poorly on the YPDA+0.4 M NaCl than on the YPDA devoid of 0.4 M NaCl at both temperatures, compared with the wild type (Figure 4.10). These results suggested that WdPacCp played a role in the regulation of Na^+ stress, which was not dependent on temperature of incubation.

4.36 The *wdpacc*$^{+/-}$*-1* mutant and *wdpacc*^{c}*-1*mutant are not affected by SDS or caffeine

Sodium dodecyl sulfate (SDS), a cell wall perturbation reagent, and caffeine, a drug that affects parts of a potential cell wall integrity pathway, are reported to inhibit the growth of the *S. cerevisiae rim101*Δ mutant (Castrejon et al., 2006). To study whether these conditions also affected the growth of the *WdPACC* mutation strains, cells were diluted and spotted on YPDA+0.002% SDS and YPDA+4 mM caffeine. The results showed that these treatments did not more reduce the growth of *WdPACC* mutants to any greater extent than that of the wild type at 25°C and 37°C (Figure 4.11).

4.37 *WdPACC* mutation affects *W. dermatitidis* sclerotic form growth at acidic pH

In pH 2.5 MCDB at 25°C, *W. dermatitidis* is induced to form sclerotic morphotypes (Wang and Szaniszlo, 2000). To investigate the effects of *WdPACC* mutation on the induction of these morphotypes, cells were cultured in pH 2.5 MCDB. As expected, at this pH, wild type cells were induced to produce sclerotic cells and some planate cells, whereas the *WdPACC* constitutive mutation repressed the conversion to planate cells. In addition, many of the *wdpacc^{c}-1* cells lost cell wall integrity and burst (Figure 4.12). Also, *WdPACC* loss-of-function mutation did not affect the conversion to the sclerotic morphotypes (data not shown). To further investigate the growth of *wdpacc^{c}-1* cells at acidic pH, cells were cultured in the pH 3 in stead of the pH 2.5 MCDB. In this case, the wild type and *wdpacc$^{+/}$-1* again generated sclerotic forms, and the *WdPACC* constitutive mutation still repressed sclerotic form growth. Interestingly, all of the *wdpacc^{c}-1* cells swelled more than those of the wild type, implying that channels were open and solutes

and water had penetrated into the cells (Figure 4.13). In pH 4.5 MCDB, the wild type, *wdpacc^{c}-1* and *wdpacc$^{+/-}$-1* grew as normal budding yeast cells (data not shown).

4.38 Northern analysis shows *WdPACC* mutation affects chitin synthase RNA expression

Because *WdCHS3* and *WdCHS5* are differentially expressed when cells were cultured for sclerotic form growth in pH 2.5 MCDB and when cells were cultured for yeast growth in pH 6.5 MCDB (Wang and Szaniszlo, 2000)(Liu et al., 2004), to further study the regulation of pH to *WdCHS* RNA expression, Northern analysis was carried out with RNA prepared from cells of the wild type, *wdpacc$^{+/-}$-1* and *wdpacc^{c}-1* cultured in pH 7 MCDB and in pH 2.5 MCDB. The results showed that *WdCHS5* RNA expression levels were similar in the wild type, *wdpacc$^{+/-}$-1* and *wdpacc^{c}-1* under each condition, indicating *WdCHS5* was not obviously regulated by the *WdPACC* mutations. *WdCHS5* RNA expression levels in the three strains were also similar when cells were cultured in pH 7 MCDB and in pH 2.5 MCDB. However, the *WdCHS5* RNA level was increased when cells were cultured in pH 2.5 MCDB from day one to day three (Figure 4.14A). These results suggested that *WdCHS5* level was mainly increased by incubation time at pH 2.5 but not by *WdPACC* mutations. WdChs5p may be required for the sustained growth of *W. dermatitidis* at pH 2.5. Also, *WdCHS3* RNA expression was not dependent on WdPacCp, although it was regulated by different pH (data not shown). In addition, *WdCHS2* and *WdCHS4* RNA expression was not controlled by pH and WdPacCp (data not shown).

In contrast, *WdCHS1* (class II chitin synthase gene) RNA expression was affected by *WdPACC* mutation. Northern analysis carried out in MCDB at pH 2.5 and pH 7 showed that *WdCHS1* expression was not obviously regulated by pH 2.5 and pH 7, and also was not obviously regulated by the *WdPACC* loss-of-function mutation at pH 7 (data not shown). However, *WdCHS1* expression was obviously decreased in *wdpacc^{c}-1* compared with the wild type at pH 2.5 (Figure 4.14B).

4.39 WdPacCp regulates yeast-hyphal transitions

In some yeast fungi, Rim101p is involved in dimorphic switching between yeast and hyphae. Whether WdPacCp also regulates yeast-hyphal transitions in *W. dermatitidis* was studied. Because WdStuAp regulated the convoluted colony surface growth on YPDA at 37°C, I first investigated whether the colony morphology of *WdPACC* mutation strains was similarly affected under this condition. At 37°C, convoluted colony surfaces were not induced in either *wdpacc$^{+/-}$-1* or *wdpacc^{c}-1* under these conditions (data not shown).

To test whether *WdPACC* regulates filamentous growth, yeast cells were spotted on PDA and incubated at 25°C and 37°C. The *wdpacc^{c}-1* was slightly more filamentous on the colony surface than the wild type at both temperatures (Figure 4.15). However, at 25°C, filamentous growth was largely repressed in *wdpacc$^{+/-}$-1* in the manner of that of the *wdstuaΔ1A* mutant (Figure 4.15). At 37°C, the repression of filamentous growth in *wdpacc$^{+/-}$-1,* like in *wdstuaΔ1A,* was only reduced, and the colony surface of *wdpacc$^{+/-}$-1*

was more filamentous than that of *wdstuaΔ1A* (Figure 4.15). To further test the difference between *wdpacc*$^{+/-}$*-1* and *wdstuaΔ1A,* cells were spread on a block on PDA and incubated at 25°C. The *wdpacc*$^{+/-}$*-1* mutant and the *wdstuaΔ1A* mutant was found to produce very a few filaments at its edges even after 7 days of incubation, whereas the wild type and *wdpacc*^{c}*-1* produced many filaments at their edges at 4 days of incubation. However, the repression of filamentous growth in *wdstuaΔ1A* and *wdpacc*$^{+/-}$*-1* was different: the filaments produced by *wdstuaΔ1A* were thicker, suggesting that different repression mechanisms of filamentous growth were operative in the *wdstuaΔ1A* and *wdpacc*$^{+/-}$*-1* mutants (Figure 4.16). Moreover, observations of PDA slide cultures revealed that many aerial hyphae and conidia were produced by the wild type, *wdpacc*$^{+/-}$*-1* and *wdpacc*^{c}*-1*, while *wdstuaΔ1A* was strongly repressed in aerial hyphal growth and conidiation (Figure 4.17). Thus, on PDA at 25°C, WdPacCp was a positive regulator of *W. dermatitidis* filamentous growth, but not a critical regulator for *W. dermatitidis* conidiation.

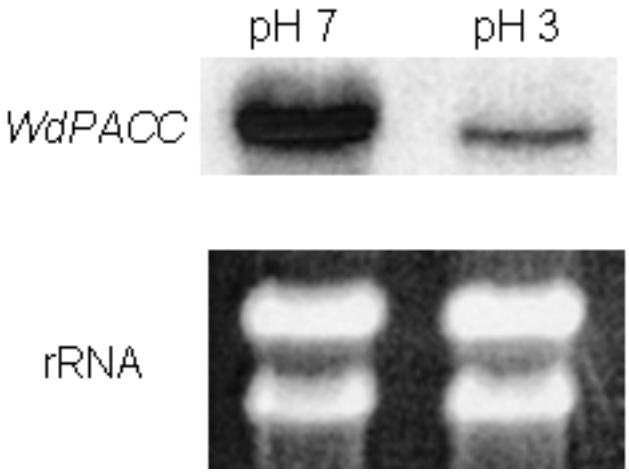
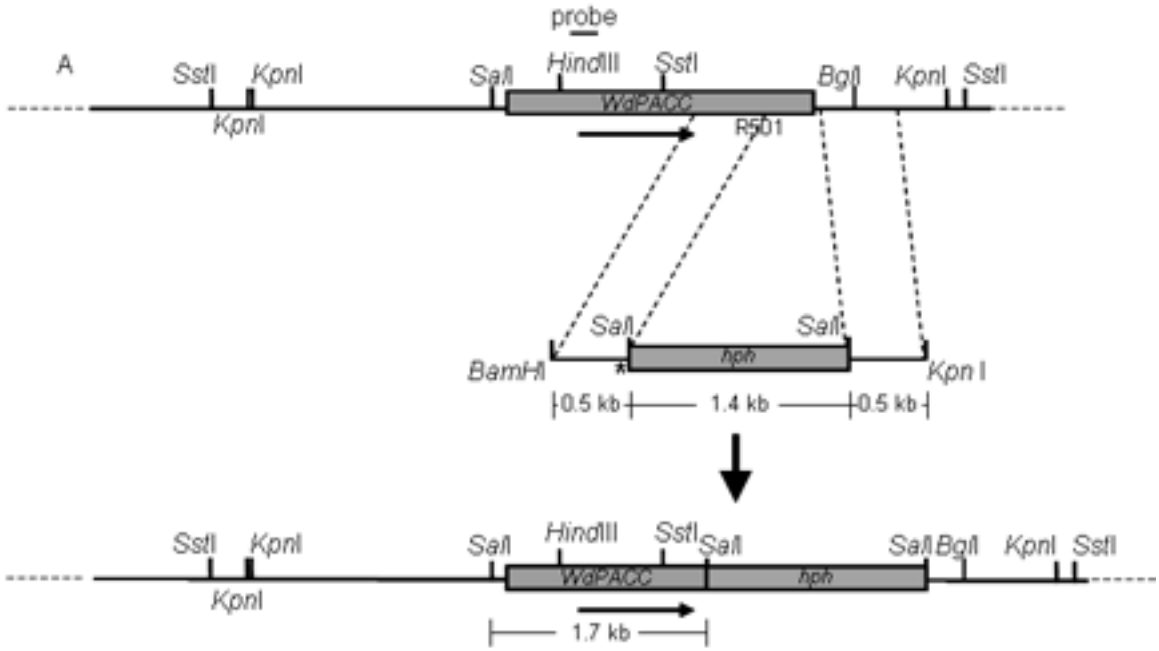


FIGURE 4.6. NORTHERN ANALYSIS OF *WDPACC* EXPRESSION. Total RNA was prepared from wild-type cells grown in pH 7 and pH 3 MCDB liquid media at 25°C for 24 h. 15 µg RNA was loaded and hybridized with a *WdPACC* probe.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KpnI
KpnI
SalI
HindIII
SstI
WdPACC
R501
BglI
KpnI
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SalI
hph
BamHI
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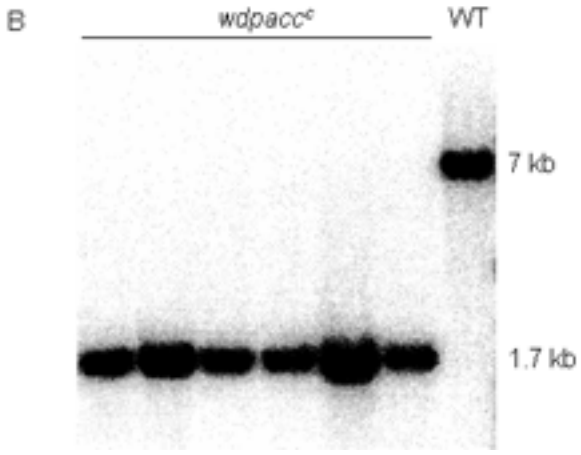
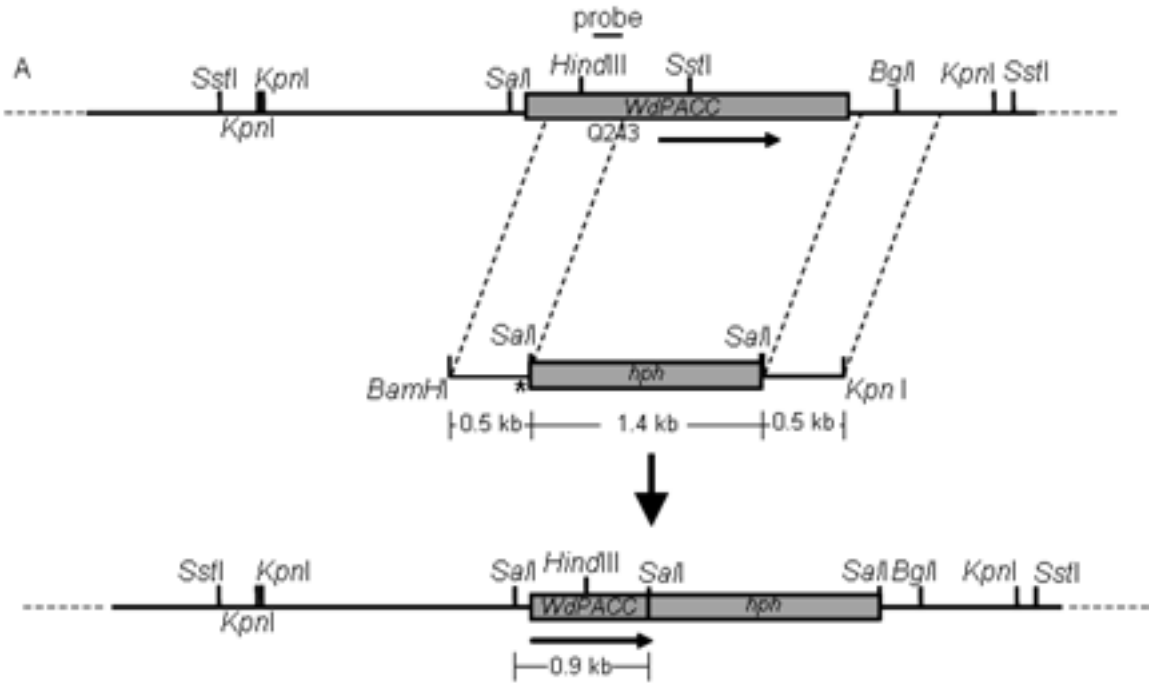

B
wdpacc^{c}
WT
7 kb
1.7 kb

FIGURE 4.7. CONSTRUCTION OF THE *WDPACC* CONSTITUTIVE MUTATION BY A ONE-STEP GENE REPLACEMENT METHOD. Strategy for the construction of *wdpacc*^{c}. A 0.5-kb 5' fragment and a 0.5-kb 3' fragment were constructed to flank the *hph* marker. This fragment was transformed into *W. dermatitidis* to produce a recombination with genomic DNA. A stop codon was introduced at the end of the 5' sequences after R501, which is denoted by a star. (C). Southern analysis. Genomic DNA of the wild-type strain and mutation strains were digested with *Sal*I and electrophoresed on a 0.8% agarose gel. DNA was hybridized with the 204-bp *WdPACC* probe. The sizes of the hybridization bands are indicated.


probe
A
SstI KpnI
KpnI
SalI HindIII SstI
WdPACC
Q243
BglI KpnI SstI
SalI
SalI
hph
BamHI
*
Kpn I
0.5 kb
1.4 kb
0.5 kb
SstI KpnI
KpnI
SalI HindIII SalI
WdPACC
hph
SalI BglI KpnI SstI
0.9 kb

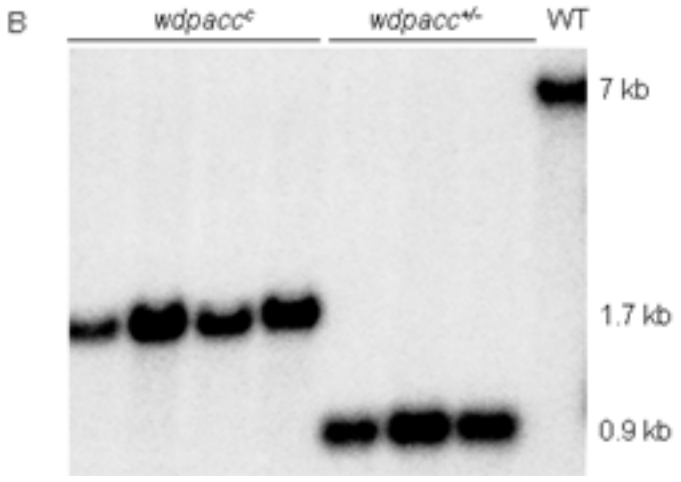

B
wdpacc-
wdpacc+/-
WT
7 kb
1.7 kb
0.9 kb

FIGURE 4.8. CONSTRUCTION OF THE *WDPACC* LOSS-OF-FUNCTION MUTATION BY A ONE-STEP GENE REPLACEMENT METHOD. Strategy for the construction of *wdpacc*$^{+/-}$. A 0.5-kb 5' fragment and a 0.5-kb 3' fragment were constructed to flank the *hph* marker. This fragment was transformed into *W. dermatitidis* to produce a recombination with genomic DNA. A Stop codon was introduced at the end of the 5' sequences after Q243, which is denoted by a star. (C). Southern analysis. Genomic DNA of the wild-type strain and mutation strains were digested with *Sal*I and electrophoresed on a 0.8% agarose gel. DNA was hybridized with the 204-bp *WdPACC* probe. The sizes of the hybridization bands are indicated.

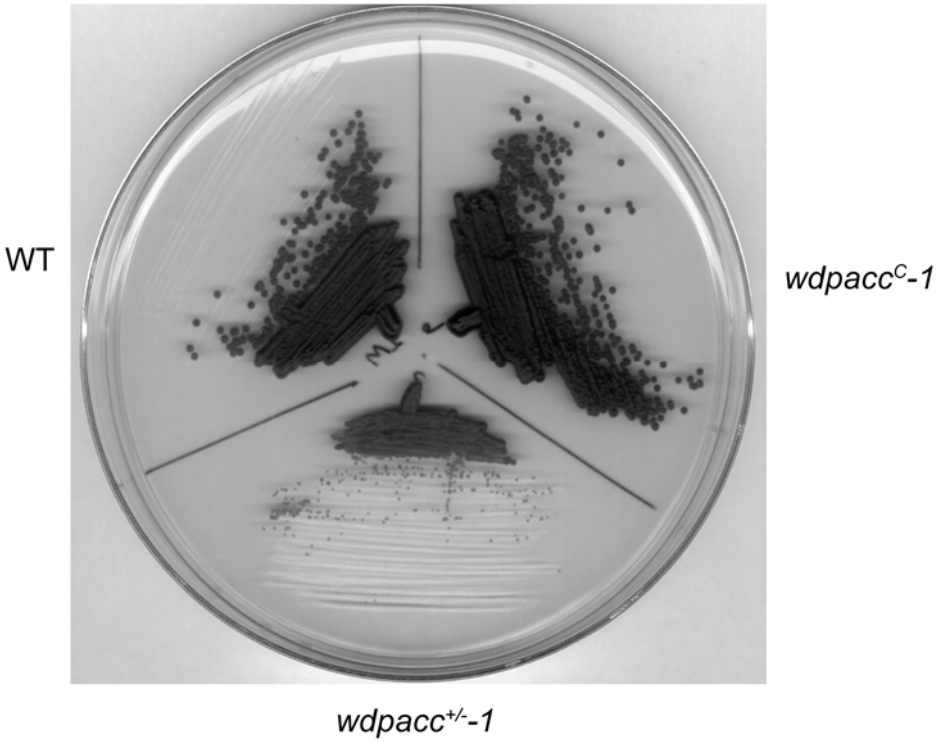


FIGURE 4.9. THE *WDPACC*$^{+/-}$*-1* GREW SLOWER AT THE NORMAL CONDITION. Yeast cells of the WT, *wdpacc*^{c}*-1* and *wdpacc*$^{+/-}$*-1* were streaked on YPDA and incubated at 25°C for 5 days.

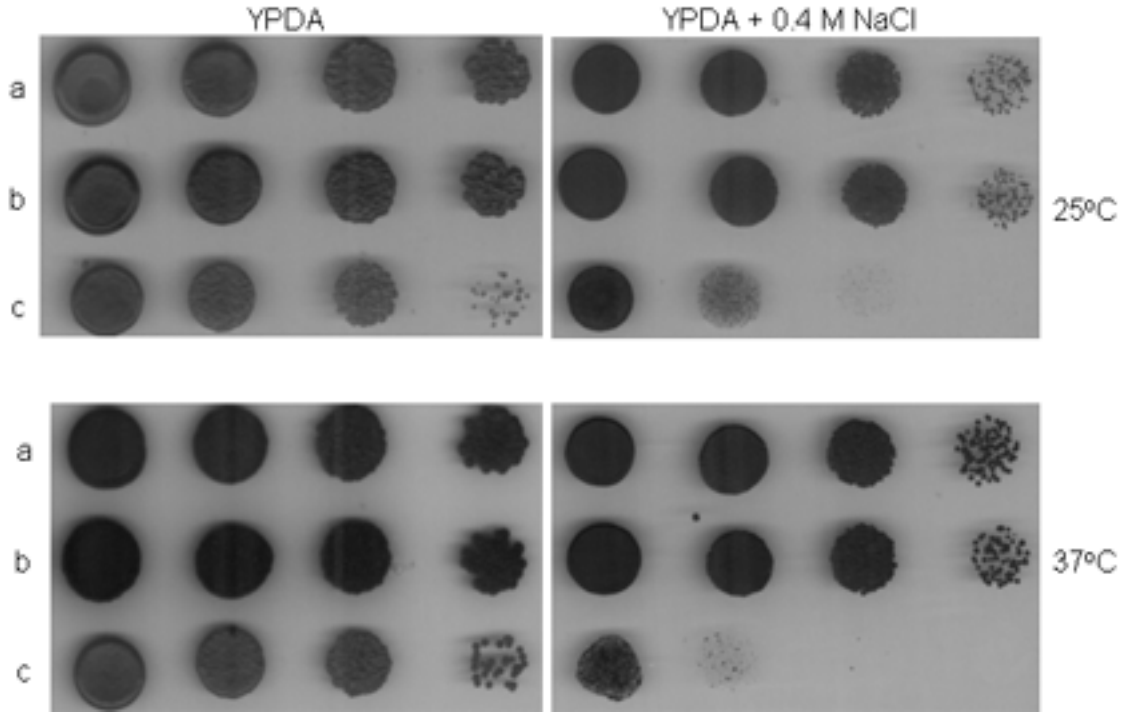


FIGURE 4.10. WDPACCP REGULATED NA^+ STRESS IN *W. DERMATITIDIS*. Spot assays. WT (a), *wdpacc*^{c}*-1* (b) and *wdpacc*$^{+/-}$*-1* (c) were spotted with 10^5, 10^4, 10^3, and 10^2 cells on YPDA and YPDA supplemented with 0.4 M NaCl, and then incubated at 25°C and 37°C for 4 days.

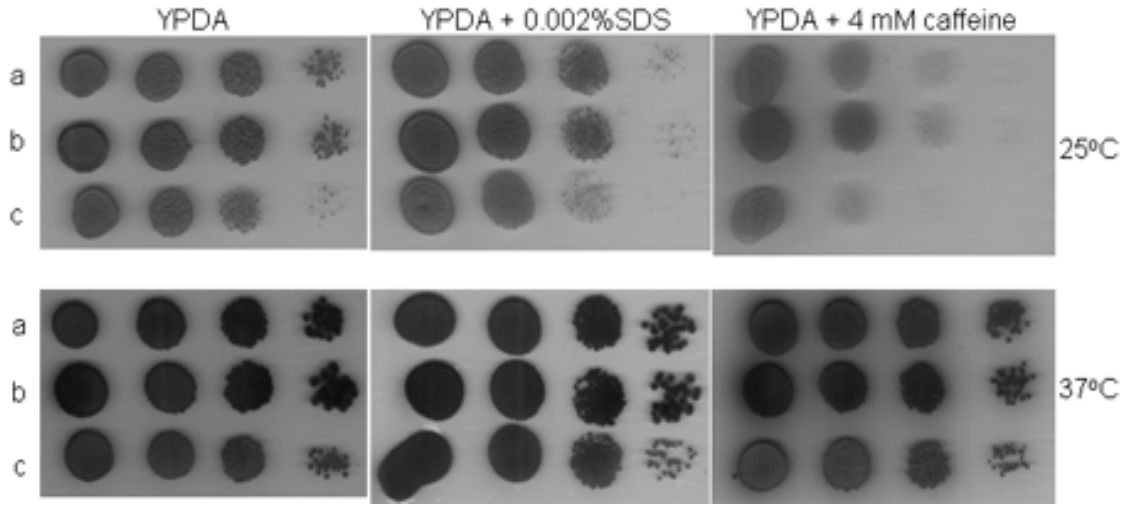


FIGURE 4.11. SPOT ASSAYS ON YPDA CONTAINING SDS AND CAFFEINE. WT (a), *wdpacc*^{c}*-1* (b) and *wdpacc*$^{+/-}$*-1* (c) were spotted with 10^5, 10^4, 10^3, and 10^2 cells on YPDA and YPDA supplemented with 0.002% SDS or 4 mM caffeine, and then incubated at 25°C and 37°C for 4 days. Note that on YPDA supplemented with 4 mM caffeine, the spots of 10^5 cells of the WT and *wdpacc*^{c}*-1* secreted many brown pigments on the agar.

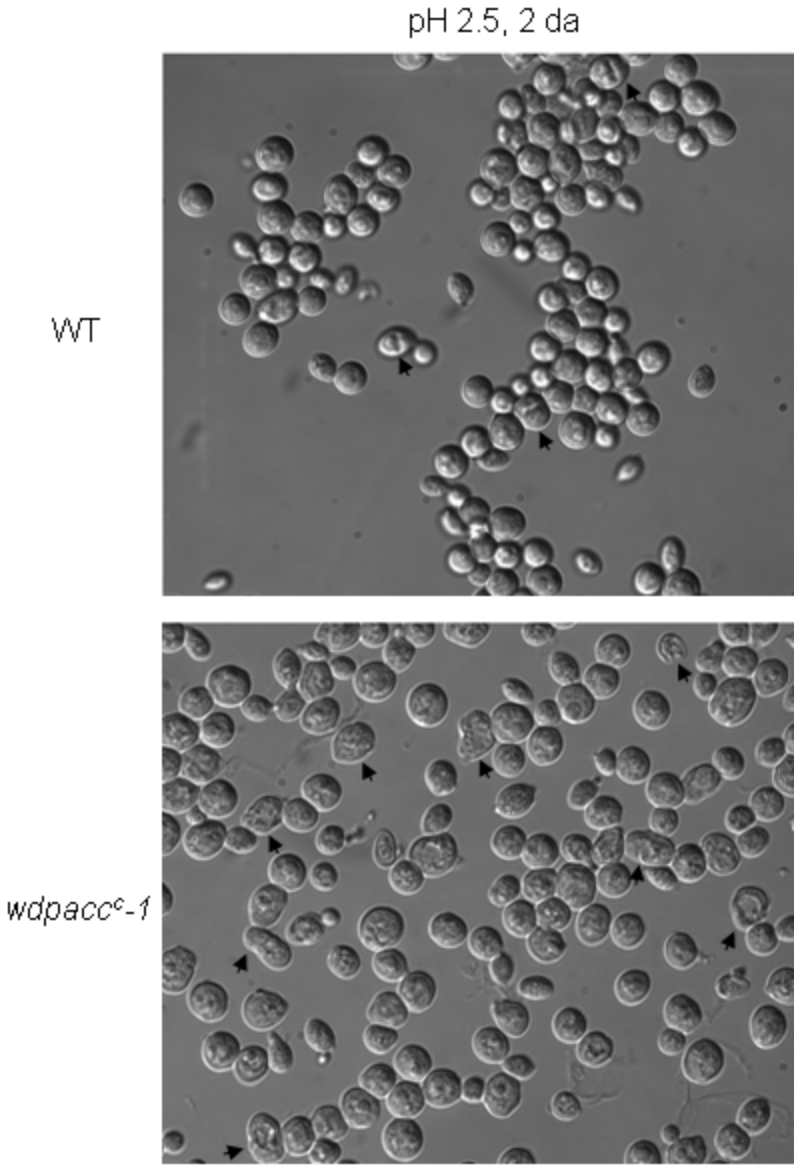


FIGURE 4.12. WT AND *WDPACC^{C}-1* WERE GROWN IN PH 2.5 MCDB AT 25°C. After 2 days, cells were photographed with a light microscope fitted with a 100x oil objective. Representative lysed cells in *wdpacc^{c}-1* and planate cells in the WT are indicated by arrows.

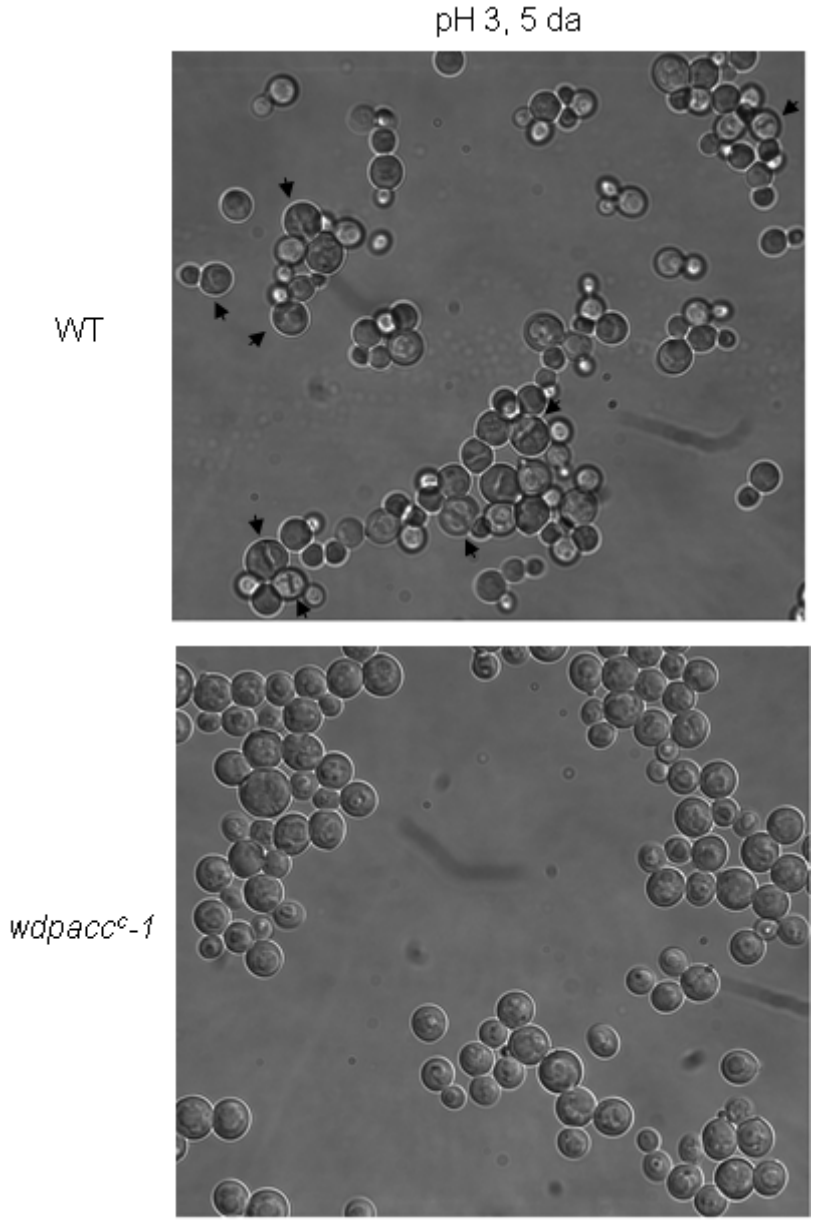


FIGURE 4.13. WT AND *WDPACC^C-1* WERE CULTURED IN PH 3 MCDB AT 25°C. After 5 days, cells were photographed with a light microscope fitted with a 100x oil objective. Representative planate cells in the WT are indicated by arrows.

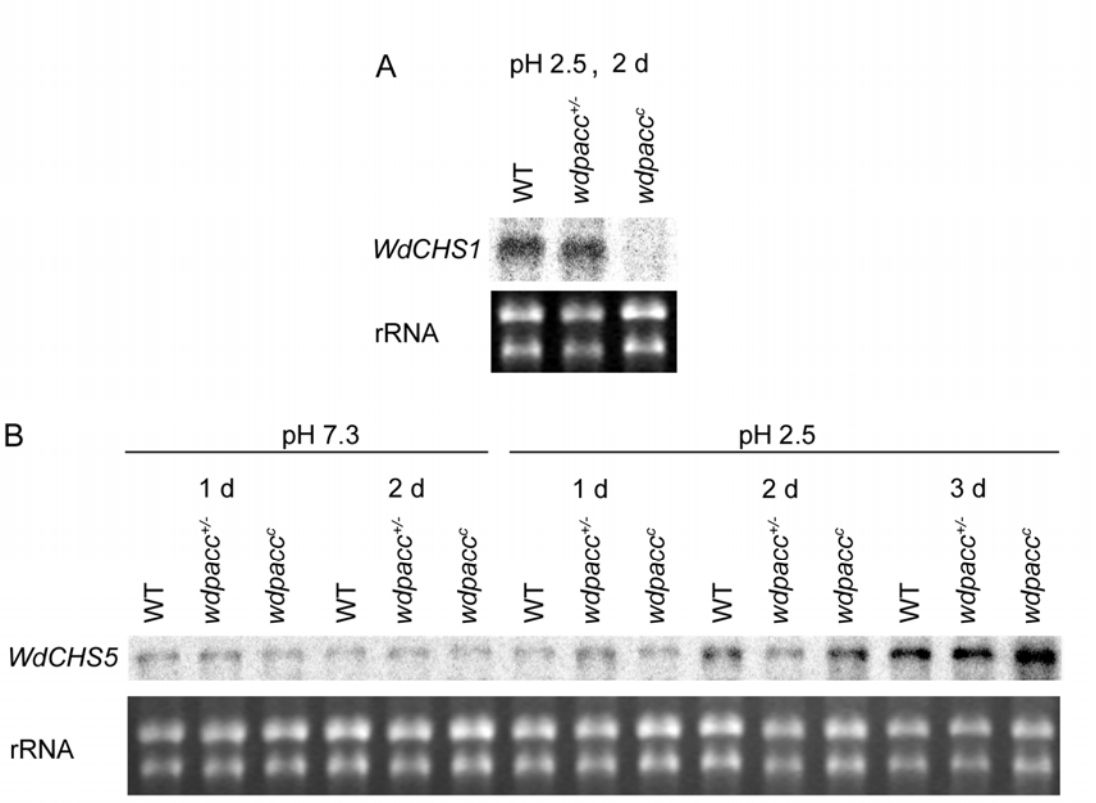


FIGURE 4.14. NORTHERN ANALYSIS OF *WDCHS5* AND *WDCHS1* EXPRESSION IN THE WT AND *WDPACC* MUTATION STRAINS. (A) Northern analysis for *WdCHS5* transcript level. Total RNA was prepared from cells grown in MCDB at pH 7 and pH 2.5 at 25°C for the days indicated. (B) Northern analysis for *WdCHS1* transcript level. Total RNA was prepared from cells grown in pH 2.5 MCDB at 25°C for 2 days. 10 μg RNA was loaded and hybridized with a *WdCHS1* probe.

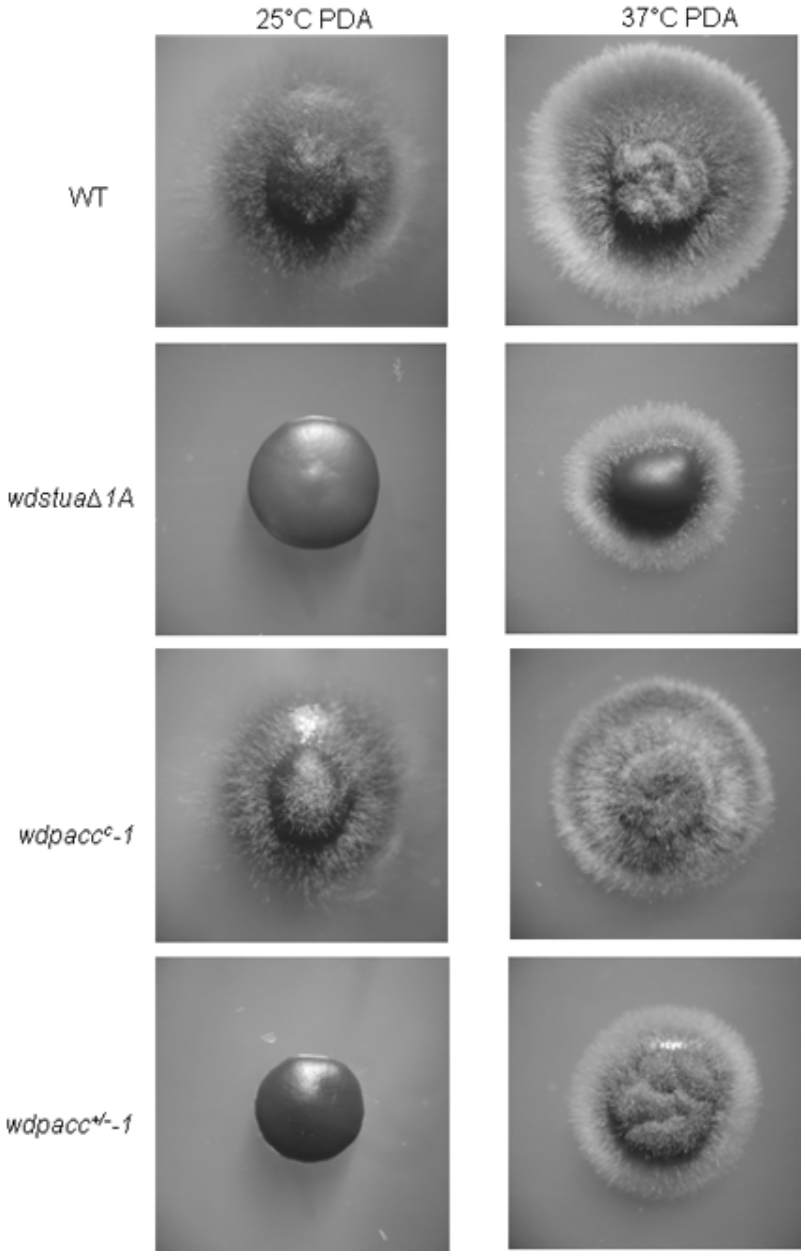


FIGURE 4.15. WDPACCP REGULATED FILAMENTOUS GROWTH IN *W. DERMATITIDIS*. WT, *wdstuaΔ1, wdpacc*^{c}*-1* and *wdpacc*$^{+/-}$*-1* were grown on PDA at 25°C and 37°C. After 8 days of incubation, colonies were photographed with a camera fitted to a dissecting microscope.

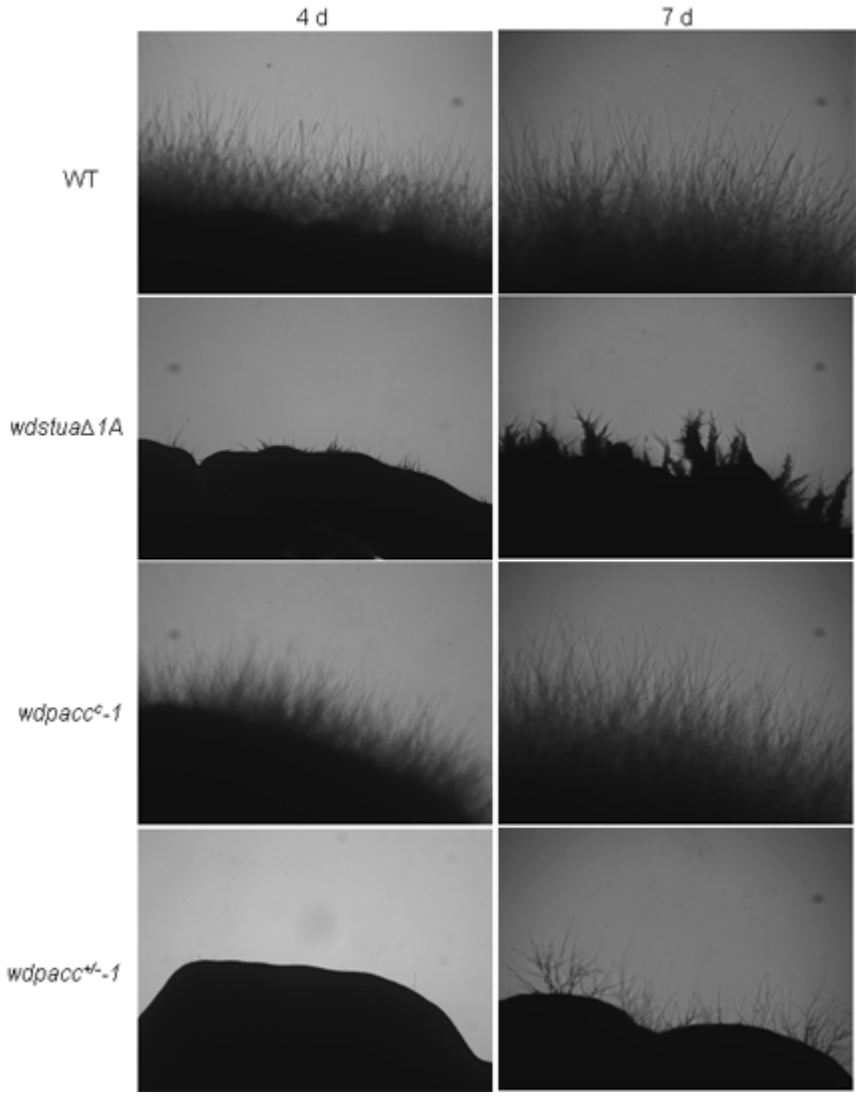


FIGURE 4.16. COMPARISON OF THE EFFECTS ON FILAMENTOUS GROWTH BY *WDSTUAΔ1* AND *WDPACC*$^{+/-}$*-1*. Yeast cells of the WT, *wdstuaΔ1, wdpacc*^{c}*-1* and *wdpacc*$^{+/-}$*-1* were spread in a block on PDA, respectively, and then incubated at 25°C. At the times indicated, colony edges were photographed with a light microscope fitted with a 5 x objective.

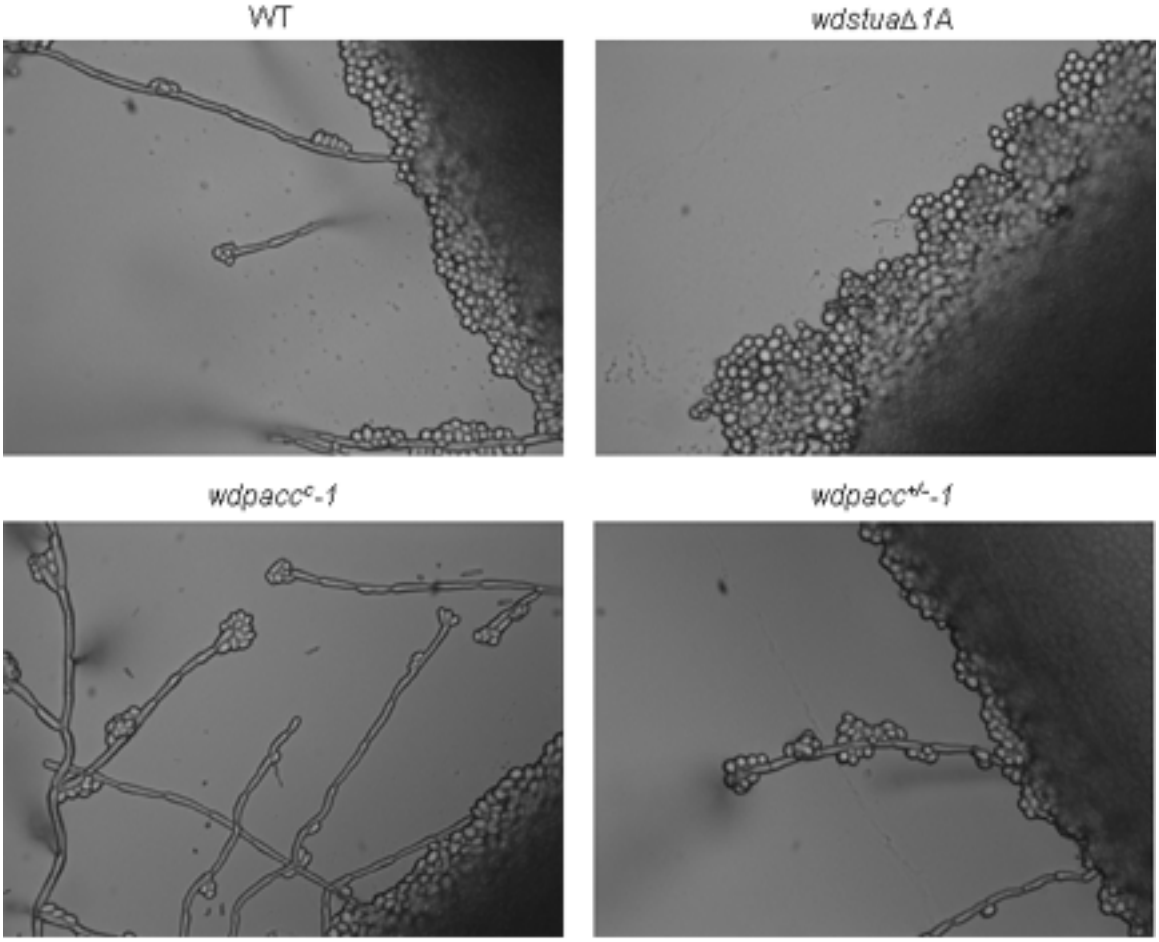


FIGURE 4.17. THE *WDPACC*$^{+/-}$*-1* MUTANT DID NOT BLOCK CONIDIOGENESIS. WT, *wdstuaΔ1, wdpacc*^{c}*-1* and *wdpacc*$^{+/-}$*-1* were inoculated on PDA in slide cultures and incubated at 25°C. After two weeks of incubation, conidiophores and conidia were photographed with a compound light microscope fitted with a 63 x objective.

4.4 DISCUSSION

W. dermatitidis can grow in a wide range of pH conditions. To survive different pH environments, *W. dermatitidis* need to adapt and respond. At pH 2.5, *W. dermatitidis* grows in sclerotic forms and its budding yeast growth is repressed. To study the growth of *W. dermatitidis* at different pH conditions, *WdPACC* gene, which encodes an important pH pathway transcription factor, was cloned. Comparison of the deduced protein sequence of WdPacCp showed that it was very conserved among PacCp family members, and had the functional motifs of AnPacCp (Figure 4.5). The three zinc finger DNA binding motifs were at the N terminus, and the signaling protease cleavage site for the activation of the protein was at the C terminus. The *WdPACC* gene contained two introns (Figure 4.4). The position of the first intron was very conserved (Caracuel et al., 2003; Flaherty et al., 2003; MacCabe et al., 1996; Rollins and Dickman, 2001; Schmitt et al., 2001; Suarez and Penalva, 1996; Tilburn et al., 1995). However, the position of the second intron was unique. Because the second intron does not introduce a stop codon or a frame shift, the existance of a regulatory mechanism of alternative splicing is suggested. Also, the *PACC* genes are suggested to be regulated by PacCp proteins, because several PacCp binding sites exist upstream of the translation start codons (Caracuel et al., 2003; Flaherty et al., 2003; MacCabe et al., 1996; Rollins and Dickman, 2001; Schmitt et al., 2001; Suarez and Penalva, 1996; Tilburn et al., 1995). Consistently, three PacCp binding sites in *WdPACC* were found to exist between -796 bp

and -1122 bp upstream of its translation start codon, as predicted by MatInspector software, with core similarities 1.000 and matrix similarities above 0.900 (Figure 4.4).

Northern analysis showed that the RNA expression of *WdPACC* was obviously higher at neutral-alkaline pH versus acidic pH (Figure 4.6). At neutral-alkaline pH and acidic pH, *WdPACC* RNA level was higher in *wdpacc^{c}-1* than in the wild type, and lower in *wdpacc$^{+/-}$-1* than in the wild type (data not shown). The *wdpacc^{c}-1* mutant grew poorly at acidic pH (Figure 4.12, 4.13), and the *wdpacc$^{+/-}$-1* mutant grew poorly at neutral-alkaline pH (data not shown). These results are in agreement with the effects of *PACC/RIM101* family members (Penalva and Arst, 2004). The *wdpacc$^{+/-}$-1* grew slower under normal cellular conditions (Figure 4.9), a result not found with the corresponding *anpacc$^{+/-}$* mutant of *A. nidulans*. The *anpacc*Δ null mutant is reported to grow poorly at permissive temperature (Tilburn et al., 1995), but *scrim101*Δ of *S. cerevisiae*, *carim101*Δ of *C. albicans* and *umrim101*Δ of Basidiomycete *Ustilago maydis* are not found to be affected in growth rate at permissive temperature (Arechiga-Carvajal and Ruiz-Herrera, 2005; Davis et al., 2000b; Li and Mitchell, 1997). Interestingly, *scrim101*Δ is cold sensitive, growing slower at 17°C (Su and Mitchell, 1993a). The mechanism of the slow growth of the *wdpacc$^{+/-}$-1* mutant is not known yet.

The *wdpacc$^{+/-}$-1* mutant showed more sensitivity to Na^{+} stress on YPDA (Figure 4.10). When different concentrations of NaCl, 0.1 M, 0.4 M and 1 M NaCl, were tested, the wild type, *wdpacc^{c}-1,* and *wdpacc$^{+/-}$-1* all were not able to grow on YPDA+1 M NaCl.

The 0.1 M NaCl and 0.4 M NaCl both reduced the growth of *wdpacc*$^{+/-}$-1 more than that of the wild type, while 0.1 M NaCl had weaker effects on reducing growth between the *wdpacc*$^{+/-}$*-1* and wild type than 0.4 M NaCl. WdPacCp may regulate the plasma membrane Na^+-ATPase pump, as reported from some fungi PacCp/Rim101p proteins (Caracuel et al., 2003; Lamb et al., 2001). Although the *wdpacc*^{c}*-1* did not show more resistance to Na^+ stress on YPDA at pH 4.5, it may show more resistance to Na^+ stress at higher pH.

The *wdpacc*^{c}*-1* mutation caused repression of sclerotic form growth, swelling when cultured at pH 3 and bursting when cultured at pH 2.5 (Figure 4.12, 4.13), while the *wdpacc*$^{+/-}$*-1* mutation did not affect sclerotic form development. In *wdpacc*^{c}*-1,* the expression of some plasma membrane pumps may be activated, so that water and solute entered the cells. To our knowledge, these morphologies of the *wdpacc*^{c}*-1* mutant at extreme low pH levels are the first report among *PACC/RIM101* constitutive mutants.

CaCHS1 (class II chitin synthase gene) RNA expression is largely decreased in *carim101*Δ at pH 7 compared with the wild type (Lotz et al., 2004). *CaCHS1* is an essential gene in *C. albicans* and required for septum formation (Munro et al., 2001). Although *WdCHS1* (class II chitin synthase gene) RNA expression was not obviously decreased in *wdpacc*$^{+/-}$*-1* at pH 7 relative to the wild type, at pH 2.5, the *WdCHS1* RNA level was clearly decreased in *wdpacc*^{c}*-1* compared with the wild type (Figure 4.14B). *WdCHS1* is critical for normal septum formation and cell wall integrity (Zheng et al., 2006). The *wdchs1*Δ mutant also grew poor at pH 2.5 (data not shown). The

decreased *WdCHS1* expression may have a relationship to the poor growth of *wdpacc^{c}-1*. PacCp/Rim101p family may directly or indirectly regulate the expression of class II chitin synthase. *WdCHS5* is an important chitin synthase gene and is activated at different stress conditions, including sclerotic form growth at pH 2.5 for 3 days versus yeast growth at pH 6.5 for 1 day (Liu et al., 2004). Northern analysis with time course revealed that *WdCHS5* expression was not mainly regulated by pH and WdPacCp, but was by culture duration at pH 2.5 (Figure 4.14A). This suggested that WdChs5p was crucial for the synthesis of chitin at the late phase growth of *W. dermatitidis* at pH 2.5.

In *S. cerevisiae,* Rim101p is required for invasive growth (Li and Mitchell, 1997). In *C. albicans, RIM101* constitutive expression activates filamentous growth at acidic pH and neutral-alkaline pH, and *RIM101* deletion represses the filamentous growth (El Barkani et al., 2000; Lotz et al., 2004). The *wdpacc^{c}-1* mutant was slightly more filamentous than the wild type (Figure 4.15). On YPDA (pH 4.5) at 25°C, after 10 days of incubation, the surface center of *wdpacc^{c}-1* had small wrinkles, while the wild type did not, suggesting that filamentous growth was activated in *wdpacc^{c}-1* (data not shown). Also when the transformants were grown on PDA (pH 5.6) at 25°C, the *wdpacc^{c}* strains can be primarily identified by having a more filamentous phenotype. The *wdpacc$^{+/-}$-1* mutant was largely decreased in filamentous growth on PDA at 25°C (Figure 4.15, 4.16). After seven days of incubation, *wdpacc$^{+/-}$-1* still grew very few filaments, while the wild type grew many filaments at four days of incubation (Figure 4.16). By comparison, on PDA at 37°C, the wild type grew many filaments at three days of incubation, while *wdpacc$^{+/-}$-1* grew comparable filaments at four days of incubation (data not shown). WdPacCp

involvement in the regulation of filamentous growth is the first report in filamentous fungi PacCp family members.

The *wdpacc*$^{+/-}$*-1* mutant and the *wdstua*Δ*1A* mutant were very similar in the repression of filamentous growth: on PDA at 25°C, they both were repressed in filamentous growth, while 37°C suppressed the repression of filamentous growth. However, the difference between *wdpacc*$^{+/-}$*-1* and *wdstua*Δ*1A* was observed. There were more aerial filaments on the colony surface of *wdpacc*$^{+/-}$*-1* than on that of *wdstua*Δ*1A* (Figure 4.15). The filaments produced by *wdstua*Δ*1A* were thicker than those produced by *wdpacc*$^{+/-}$*-1* (Figure 4.16). Slide culture on PDA at 25°C showed that *wdpacc*$^{+/-}$*-1* grew many conidiophores and conidia, while *wdstua*Δ*1A* was strongly repressed in aerial hyphal growth and conidiation (Figure 4.17). These findings suggested that although WdStuAp and WdPacCp both may regulate hypha-specific genes, WdStuAp may also regulate genes required for the growth of aerial hyphae and conidiophores, whereas WdPacCp may not. In addition, WdStuAp was a negative regulator of convoluted colony surface growth on YPDA at 37°C, while WdPacCp did not have obvious effects to this phenotype. In *C. albicans*, Efg1p and Rim101p both regulate hyphal-specific genes, such as *HWP1* (El Barkani et al., 2000; Lotz et al., 2004), and Efg1p is not required for the regulation of some Rim101p regulated genes (El Barkani et al., 2000). Further exploring the mechanism of WdPacCp and WdStuAp regulating filamentous growth and possible virulence in *W. dermatitidis* is anticipated.

In summary, my research suggests that WdPacCp is an important regulator of response to ambient pH, which helps *W. dermatitidis* survive in the different pH environments and infect host niches with their different pH. WdPacCp is first reported among PacCp orthologs of conidiogenous filamentous fungi to regulate yeast-hyphal transtions. The structure and function of *WdPACC*, the WdPacCp pathway, the protein level investigation of WdPacCp by EMSA and Western analysis, the regulation targets and virulence analysis can be further studied in the future.

Appendix A Detection of the DNA-protein binding at *WdCHS5* promoter regions

A.1 INTRODUCTION

Wangiella dermatitidis strains with *WdCHS5* alone disrupted result in loss of viability in late log and early stationary phase at 37°C, but not at 25°C, and also lose virulence in a mouse model of acute infection. *WdCHS5* is apparently up-regulated under stress conditions, such as the shift of cells to temperatures of infection, acidic pH, Ca^{++} limitation, nitrogen starvation conditions. When *W. dermatitidis* was cultured at 37°C, truncations of upstream sequences of *WdCHS5* analyzed by use of a *lacZ* reporter gene showed that *WdCHS5* contains repression and activation regions (Liu et al., 2004).

A.2 MATERIALS AND METHODS

WdCHS5 promoter fragments, R, A, R1, R2, A1, A2, S1 and D1 (Figure 1), were amplified by PCR with template pT2050 ((Liu, 2003), kindly provided by Dr. Hongbo Liu) and primers WdCHS5F2 and WdCHS5R2, WdCHS5F3 and WdCHS5R3, WdCHS5F4 and WdCHS5R4, WdCHS5F5 and WdCHS5R2, WdCHS5F3 and WdCHS5A1R, WdCHS5A2F and WdCHS5R3, WdCHS5F2 and WdCHS5S1R, WdCHS5F2 and WdCHS5D1R, (Table 1), respectively, followed by cloning into pGEM-

T easy vectors. After sequencing confirmation, fragments were cut by enzyme restriction with *Eco*RI and then gel-purified. Exonuclease-free Klenow (Ambion) and α-^{32}P dATP (Dupond Co., Wilmington, Del.) were used to label the fragments by filling in 5' overhangs. For the T1, T2, T3 and T4 fragments (Figure 1), WdCHS5T1F, WdCHS5T2F, WdCHS5T3F and WdCHS5T4F single-stranded oligos (Table 1) were labeled with γ-^{32}P dATP (Dupond Co., Wilmington, Del.) and T4 polynucleotide kinase (Fisher), followed by mixing at a 1:1 molar ratio with WdCHS5T1R, WdCHS5T2R, WdCHS5T3R and WdCHS5T4R single-stranded oligos (Table 1). Oligos were diluted to 1 pmol/µl in TEN buffer (10 mM Tris-Cl, 1mM EDTA, 50 mM NaCl, pH 8.0. The oligo mixture was incubated in a water bath at 100°C for 5 min, after which time the oligo mixture was left in the water bath to slowly cool to room temperature to generate the double-stranded oligos. The labeled DNA fragments were finally purified by phenol/chloroform extraction and Sephadex G50 filtration.

For protein isolation, *W. dermatitidis* cells were first cultured at 25°C in YPDB, then inoculated into YPDB at 10^6 cells/ml and shaken vigorously at 37°C for 24 h. After collection by centrifugation, the cells were washed with ice-cold deionized water, resuspended in four cell volumes of ice-cold glass bead disruption buffer (20 mM Tris-Cl (pH 7.9), 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 1 mM DTT, 0.3 M ammonium sulfate and 0.7 x protease inhibitor), mixed with four cell volumes of ice-cold glass beads, and intermittently destroyed by vortexing for 30 sec and cooling on ice for 1 min respectively six times. After the resulting supernatant was collected and further centrifuged for 60 min at 12,000x g at 4°C and the cell debris discarded, it was dialyzed

and stored until used at -80°C. Protein concentration was determined by Bradford method (Bio-Rad).

The binding reactions of DNA and the cell protein extracts were carried out in 20% glycerol, 20 mM Tris·Cl (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 10 mM DTT, 0.7 x protease inhibitor (Complete, mini, EDTA-free protease inhibitor cocktail tablets, Roche, Renzberg, Germany), and 0.1 μg/ul nonspecific carrier DNA poly(dI-dC)·poly(dI-dC) (Amersham Biosciences, Piscatway, NJ) at room temperature for 20 min. After electrophoresis in a 4% polyacrylamide gel, radioactive signals were detected by exposure of a phosphorimager screen, which was then scanned with the phosphorimager in the scanning facility.

A.3 RESULTS AND DISCUSSION

Electrophoretic mobility shift assay (EMSA) of putative regulatory regions in the *WdCHS5* promoter shows protein binding

Deletion analysis of the *WdCHS5* promoter fused to the *lacZ* reporter gene demonstrated that a cis-acting DNA region maps between -450 to -880 relative to the translation start codon (Liu et al., 2004). Several putative binding sites were predicted in this region. To detect protein-DNA binding in this region, EMSA with protein extracts prepared from cells grown in YPD at 37°C was carried out. The sequence from -450 to -880 was divided into two fragments R [putative repressor(s) region] and A [putative activator(s)

region] (Fig. 2A). Both fragments were found to be bound by portions of the cell extracts (Fig. 2B). Of these, one binding signal with fragment A was more intense than any of those with fragment R. To delineate the binding region further, fragment R was divided into fragment R1 and fragment R2 (Fig. 2A). EMSA showed the binding with the R fragment was located in the R1 region (Fig. 2B), which contains a dense cluster of predicted transcription factor binding sites (14). To test whether the binding was specific, competition assays were carried out with 25x and 250x specific and nonspecific competitors (Fig. 3A, B). The results demonstrated that the binding detected was reduced in every case by specific competitors, but not by nonspecific competitors. Similarly, fragment A was also divided into two portions A1 and A2 (Fig. 3C). By competition assay, the binding of fragment A was demonstrated to be in fragment A1, which has three putative TATA boxes (HCtata for TATA box search program). Because these bindings with R1 and A1 may contribute to the regulation of *WdCHS5*, cell proteins prepared from cells grown in YPD at 25°C were also assayed, while produced similar results, suggesting that the bindings were not induced by temperature shifts (data not shown). The regulation of the *WdCHS5* promoter was explored further by β-gal assay of cells cultured under different induction conditions, which included the induction by Ca^{++}, pH, or caffeine (parts of a potential cell wall integrity pathway). The results were consistent with the regulation pattern identified with 37°C YPD condition (data not shown).

To further localize the binding sites in R2, deletion and base substitution experiments were performed (Fig. 4A). Base substitution was based on the published mutation

constructs of HAP/PACC. Deletion abolished the binding band with the low mobility, while base substitution made that binding band position change (Fig. 4B). These results suggested that R2 had a binding site at the HAP/PACC region. To detect the mutation effects in vivo, mutation constructs were integrated at the nonessential *WdPKS1* genomic locus and β-galactosidase assays carried out. The results showed that the base substitution at HAP/PACC site reduced the activity of *WdCHS5* promoter by 30%, whereas base substitution at the nearby REPCAR1 site did not alter the *WdCHS5* promoter activity (data not shown), suggesting HAP/PACC site played a role in activation. In addition, annealed oligos containing a HAP/PACC site on the *WdCHS5* promoter were able to form a binding band detected by EMSA (data not shown). Moreover, to detect the binding region of A1, four overlapping fragments (T1-T4) were designed to cover the sequence of A1 (Fig. 5A). The three predicted TATA boxes are located in T1, T2 and T3, respectively. The results indicated T1 and T4 had stronger binding signals than T2 and T3 (Fig. 5B). In conclusion, protein bindings at the putative repression region and activation region of *WdCHS5* promoter were detected. However, because recent analysis of the regions revealed an open reading frame for the newly described gene DBP5 helicase, these experiments were not extended further.

Table A.1. The primers used to construct the fragments of the *WdCHS5* promoter

Primer	Sequence (5' to 3')
WdCHS5F2	GAATTCGAAGCGACCTCATCTGCC
WdCHS5R2	GAATTCCGACCAAGTCTTTCGATCC
WdCHS5F3	GAATTCTCTATAAGTTAGTGTACCTATTCTA
WdCHS5R3	GAATTCTAACGCTGTTACCGGTAC
WdCHS5F4	GAATTCTGTGCCAGAGGTAGGTTGGA
WdCHS5R4	GAATTCGTCCGCTCCTACTTCTGGA
WdCHS5F5	GAATTCGAAGTAGGAGCGGACATTGT
WdCHS5A1R	GAATTCCCCTAAAAACCGTCCCTACA
WdCHS5A2F	GAATTCTGTAGGGACGGTTTTTAGGG
WdCHS5D1R	GAATTCGGCCGACCGTATCTCCAA
WdCHS5S1R	GAATTCTCTGGAGGCGGCTCCTT*ACT*CGACCGTATCT
WdCHS5TF1	TCTATAAGTTAGTGTACCTATTCTAGAGGGTC
WdCHS5TR1	GACCCTCTAGAATAGGTACACTAACTTATAGA
WdCHS5TF2	AGGGTCTATAGAGTATTTTAATAAGTTTTCAT
WdCHS5TR2	ATGAAAACTTATTAAAATACTCTATAGACCCT
WdCHS5TF3	TTTCATAGAAGTTGAGCTTCTGGTATAGAAGTA
WdCHS5TR3	TACTTCTATACCAGAAGCTCAACTTCTATGAAA
WdCHS5TF4	GAAGTAATCACGGTTCTTGTAGGGACGGTTTTTAGGG
WdCHS5TR4	CCCTAAAAACCGTCCCTACAAGAACCGTGATTACTTC

The introduced *Eco*RI restriction digestion enzyme recognition sites are underlined. The substituted nucleotides in WdCHS5S1R primer are indicated by italic letters.

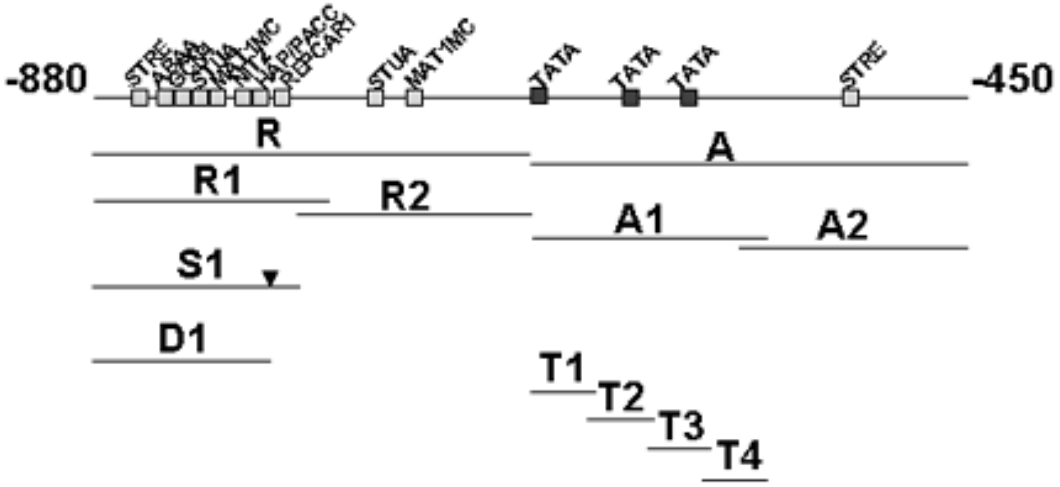


FIGURE A.1. DIAGRAM OF DNA FRAGMENTS OF THE *WDCHS5* PROMOTER USED IN EMSA. The putative cis-acting elements are noted, and the position of the substitution of nucleotides in the S1 fragment is indicated by an arrow point.

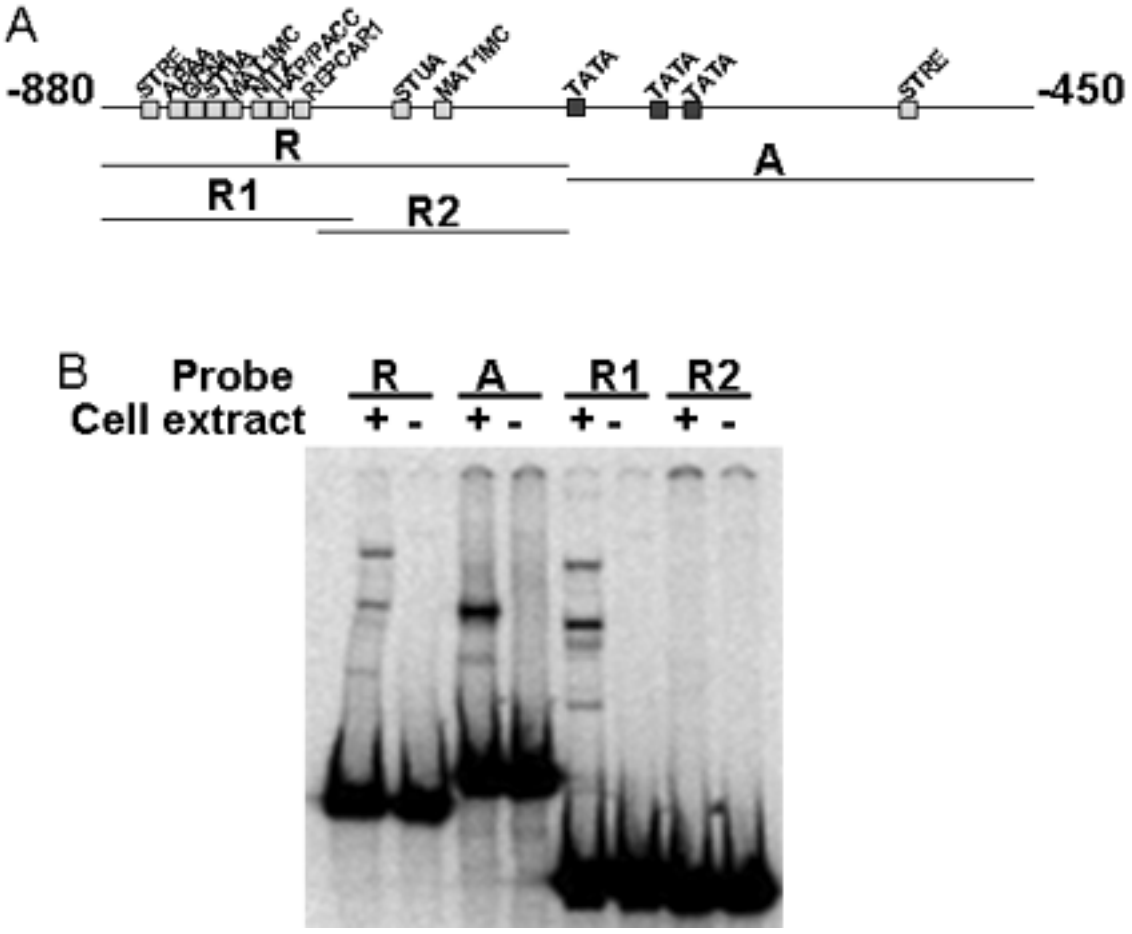


FIGURE A.2. EMSA WITH CELL EXTRACTS AND DNA FRAGMENTS OF THE *WDCHS5* PROMOTER. (A) The diagram of fragments A, R, R1 and R2. (B) Fragments A, R, R1 and R2 were used as probes in EMSA with the *W. dermatitidis* cell extracts.

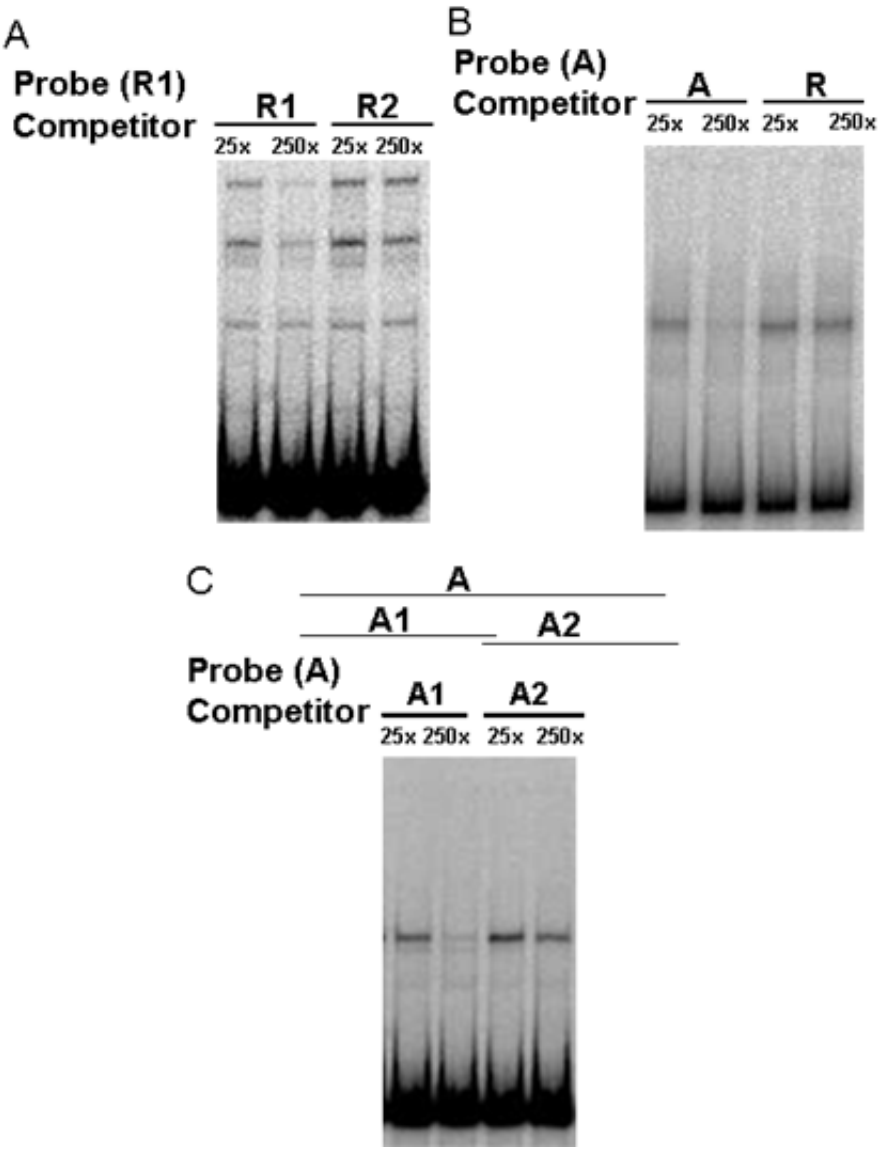


FIGURE A.3. COMPETITION ASSAYS. Using 25x, 250x of specific sequence and nonspecific sequence, the binding intensity of sequence R1 (A) and A (B) was decreased by adding a specific sequence, but not by adding a non-specific sequence. Binding in the region A was competed by the sequence A1, but not by the sequence A2, indicating that the binding was in region A1 (C).

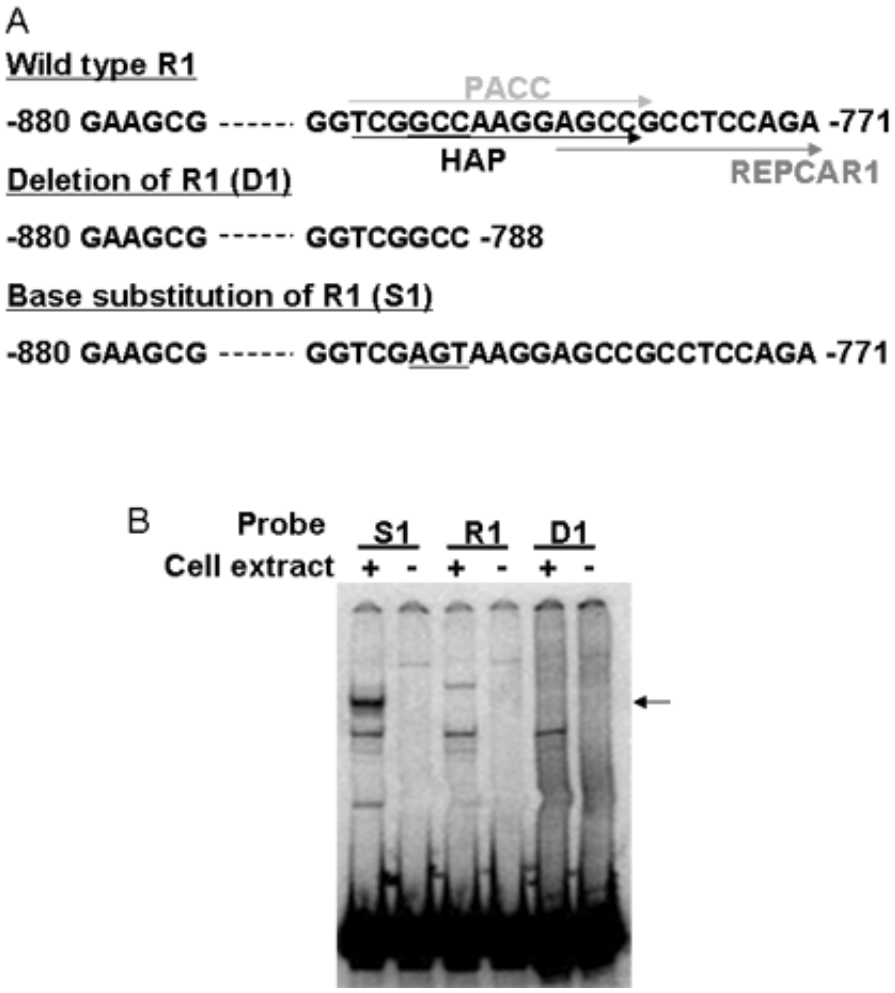


FIGURE A.4. ANALYSIS OF THE BINDING SITES USING DELETION OR BASE SUBSTITUTIONS. (A) Construct designs. Predicted binding motifs are indicated by arrows. The underlined nucleotides are mutated in the S1 fragment. (B) D1 completely abolished the protein binding of the low mobility DNA-protein complex, which is indicated by by the arrow. S1 resulted in loss in the formation of the original low mobility band and generated another band with smaller molecular weight.

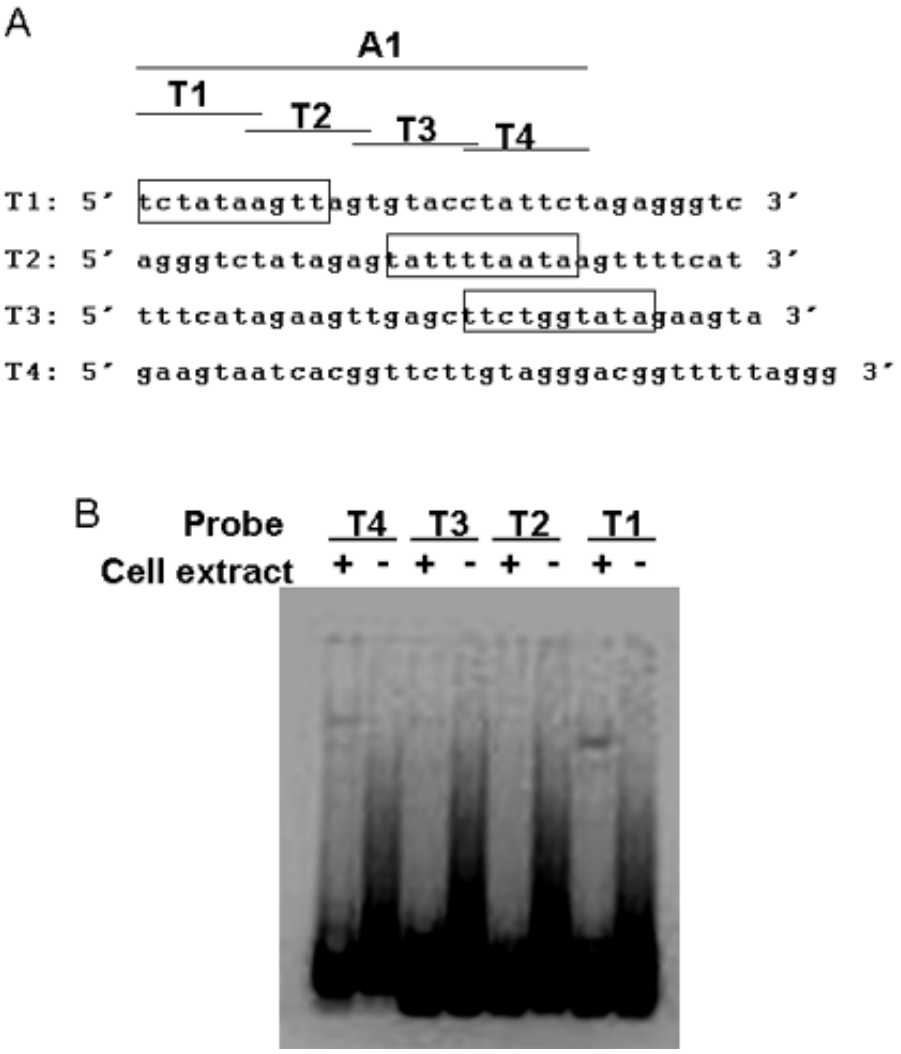


FIGURE A.5. EMSA WITH CELL EXTRACTS AND DNA FRAGMENTS IN THE A1 REGION. (A) The four fragments designed to cover the sequence A1. The three predicted TATA box binding motifs are noted by boxes. (B) Assays of binding of sequences T1 to T4 with cell extracts.

Appendix B Full list of regulated genes by the pYES2-*WdSTUA* expression strain vs the pYES2 vector alone strain

UNIQID	NAME	Log2 (test/ctrl)		
		SC26-096	SC26-098	SC26-099
IYLR255C		-0.926	-0.671	-0.206
YMR012W	CLU1	-0.805	-0.53	-0.28
IYDR286C		-1.534	-0.971	-0.795
IYOL124C		-0.983	-0.635	-0.533
YOL077W-A	ATP19	-0.526	-0.73	-0.232
IYDR126W		-0.514	-0.655	-0.234
IYLR359W		-0.598	-0.763	-0.166
YOL107W		-0.526	-0.662	-0.181
YJL161W		-0.551	-0.69	-0.11
YKL009W	MRT4	-0.56	-0.68	-0.114
IYDR358W		-0.691	-0.788	-0.117
YMR030W	RSF1	-0.608	-0.728	-0.096
IYML126C		-0.542	-0.589	-0.073
YDR532C		-0.771	-0.85	-0.236
IYKL072W		-0.754	-0.75	-0.203
YBR250W		-0.66	-0.575	-0.109
IYHL007C		-0.714	-0.571	-0.124
IYDL167C		-0.692	-0.658	-0.125
IYOR315W		-0.743	-0.574	-0.068
IYMR319C-0		-0.68	-0.662	-0.054
YIL173W	VTH1	-0.54	-0.52	-0.044
IYGL212W		-0.655	-1.399	-0.229
YGL119W	ABC1	-0.559	-1.21	-0.182
INTQ0280E		-0.698	-1.367	-0.238
IYAL010C		-0.556	-1.166	-0.104
IYDR179W-A		-0.593	-1.068	-0.355
YBR162W-A	YSY6	-0.62	-1.122	-0.327
YMR138W	CIN4	-0.588	-1.118	-0.334
IYMR089C		-0.615	-1.185	-0.286
YBR103W	SIF2	-0.519	-0.938	-0.236
YMR112C	MED11	-0.573	-1.367	-0.327
IYJL037W		-0.593	-1.266	-0.302
IYOL049W		-0.8	-1.658	-0.503
ITG(GCC)F1		-0.672	-1.497	-0.47
YKL001C	MET14	-0.66	-1.148	-0.434
YOL087C		-0.582	-0.921	-0.325
YGL247W	BRR6	-0.339	-2.38	-0.595
YLR345W		-0.602	-2.291	-0.58
IYGL051W		-1.134	-3.681	-0.371
YLL004W	ORC3	-0.526	-1.894	-0.044
Q0170		-0.691	-0.716	-0.595
YNL144C		-0.503	-0.587	-0.471
IYBR140C		-0.699	-0.74	-0.523
YDL020C	RPN4	-0.544	-0.56	-0.393

YBR049C	REB1	-0.833	-0.948	-0.603
IYDL142C		-0.7	-0.883	-0.606
YGL121C	GPG1	-0.527	-0.725	-0.452
IYDR174W		-0.512	-0.665	-0.494
YGR194C	XKS1	-0.763	-1.04	-0.76
IYEL022W		-0.641	-0.858	-0.626
YDR149C		-0.489	-0.724	-0.546
YJL074C	SMC3	-0.574	-0.949	-0.577
YGR115C		-0.547	-0.839	-0.533
YGR071C		-1.02	-1.367	-0.77
YIR010W	DSN1	-0.529	-0.645	-0.366
IYGR172C		-0.512	-0.597	-0.297
YNL295W		-0.505	-0.572	-0.28
YFL023W	BUD27	-0.509	-0.557	-0.3
IYOR330C		-0.846	-0.889	-0.393
IYLR155C-A		-0.757	-0.808	-0.357
IYJR048W		-1.43	-1.716	-0.72
YDR172W	SUP35	-0.549	-0.557	-0.303
IYBR114W		-0.542	-0.533	-0.314
YLL012W		-0.805	-0.725	-0.439
YGR286C	BIO2	-0.902	-1.003	-0.959
IYOL047C		-0.557	-0.64	-0.61
IYDL114W		-0.603	-0.694	-0.719
IYLR152C		-0.533	-0.672	-0.701
IYGR081C		-0.476	-0.592	-0.606
YFR031C	SMC2	-0.596	-0.782	-0.722
YKL088W		-0.44	-0.619	-0.563
YDR326C		-0.567	-0.576	-0.763
IYGR234W		-0.505	-0.512	-0.714
YHR066W	SSF1	-0.676	-0.57	-0.592
YDR274C		-0.819	-0.672	-0.645
LSR1		-0.86	-0.779	-0.696
IYDR501W		-0.659	-0.577	-0.511
YGR187C	HGH1	-0.296	-0.719	-0.613
YGR044C	RME1	-0.246	-0.613	-0.529
IYBL022C		-0.294	-0.706	-0.573
YOR282W		-0.274	-0.659	-0.549
YOR309C		-0.233	-0.57	-0.515
YML074C	FPR3	-0.316	-0.687	-0.577
YNL321W		-0.531	-1.1	-0.912
IYDR153C		-0.386	-0.8	-0.679
YJR133W	XPT1	-0.383	-0.745	-0.57
YHR071W	PCL5	-0.381	-0.705	-0.547
YPL089C	RLM1	-0.37	-0.69	-0.57
IYBR154C		-0.417	-0.759	-0.614
YGR114C		-0.402	-0.777	-0.64
YKL021C	MAK11	-0.438	-0.741	-0.606
YBR007C		-0.361	-0.748	-0.56
YPL138C	SPP1	-0.455	-0.704	-0.691
ICEN8		-0.358	-0.579	-0.558
YBR211C	AME1	-0.369	-0.592	-0.525

YDR454C	GUK1	-0.36	-0.572	-0.52
YLR344W	RPL26A	-0.326	-0.643	-0.615
YDR029W		-0.5	-0.905	-0.845
IYDL087C		-0.309	-0.597	-0.526
IYKL134C		-0.307	-0.84	-0.603
YDR073W	SNF11	-0.258	-0.771	-0.533
IYGR233C-1		-0.279	-0.695	-0.523
YGL117W		-0.644	-1.18	-1.285
YLR162W		-0.375	-0.674	-0.731
IYPR013C-0		-0.31	-0.544	-0.569
Q0150		-0.708	-1.406	-1.492
YML087C		-0.384	-0.836	-0.865
RDN18-1B		-0.35	-0.893	-0.902
YPL074W	YTA6	-0.307	-0.72	-0.709
YLR371W	ROM2	-0.218	-0.597	-0.707
INTQ0185C		-0.305	-0.659	-0.792
YJR150C	DAN1	-0.223	-0.541	-0.601
YHR077C	NMD2	-0.374	-0.642	-0.86
YLR189C	ATG26	-0.351	-0.608	-0.769
YBR013C		-0.359	-0.694	-0.874
YOR095C	RKI1	-0.285	-0.519	-0.668
YBL022C	PIM1	-0.336	-0.543	-0.648
YIL165C		-0.535	-0.744	-0.901
YDR244W	PEX5	-0.37	-0.549	-0.631
YDL121C		-0.585	-0.746	-0.957
YOL118C		-0.308	-1.167	-0.697
IYPR046W		-0.371	-1.312	-0.666
IYDR028C		-0.339	-1.122	-0.6
IYCR034W		-0.403	-1.189	-0.588
IYGR250C-1		-0.384	-1.252	-0.508
IYLR228C-1		-0.557	-1.196	-0.561
YCR027C	RHB1	-0.468	-1.094	-0.528
YDR210W		-0.624	-1.218	-0.582
IYHR197W		-0.371	-0.971	-0.508
YJR048W	CYC1	-0.381	-0.924	-0.513
YKR031C	SPO14	-0.453	-1.031	-0.543
YFL008W	SMC1	-0.437	-0.865	-0.537
YOR313C	SPS4	-0.53	-1.06	-0.61
IYBR135W		-0.458	-0.915	-0.521
YHR162W		-0.486	-0.994	-0.66
IYAL058C-A		-0.532	-0.949	-0.639
YMR088C		-0.301	-0.997	-0.904
YGR009C	SEC9	-0.179	-0.828	-0.697
YKL069W		-0.114	-0.583	-0.501
YHL009C	YAP3	-0.112	-0.572	-0.508
IYGL173C		-0.208	-0.961	-0.937
YKL063C		-0.068	-0.783	-0.511
YER098W	UBP9	-0.084	-0.938	-0.693
YJL058C	BIT61	-0.051	-0.785	-0.596
YHR030C	SLT2	-0.122	-0.965	-0.742
YOR210W	RPB10	-0.369	-1.702	-1.239

ISNR8		0.033	-0.625	-0.606
YLR336C	SGD1	0.008	-0.526	-0.565
IYGR279C		-0.064	-0.802	-0.88
IYDR135C		-0.084	-0.911	-0.931
YCR025C		-0.023	-0.545	-0.549
YML081W		-0.029	-0.66	-0.575
IYFR036W		-0.017	-0.656	-0.547
IYOR298C-A		0.008	-1.061	-0.942
YLR377C	FBP1	0.007	-0.591	-0.517
IYPR166C		0.067	-0.909	-0.789
YNL163C	RIA1	0.048	-0.77	-0.62
YDR425W	SNX41	-0.179	-0.55	-0.931
YPL069C	BTS1	-0.213	-0.521	-0.836
YDR358W	GGA1	-0.209	-0.533	-0.739
YGR010W	NMA2	-0.221	-0.527	-0.722
YLR169W		-0.129	-0.726	-0.891
YDR389W	SAC7	-0.155	-0.526	-0.578
YGR066C		-0.298	-1.221	-1.377
YIL151C		-0.262	-1.121	-1.268
YMR075W		-0.017	-0.556	-0.744
YER169W	RPH1	-0.039	-0.791	-1.226
IYMR250W		0.016	-0.771	-1.146
YEL039C	CYC7	0.046	-0.553	-0.705
YKL059C	MPE1	0.031	-0.533	-0.629
YNL280C	ERG24	-0.197	-1	-1.842
YNR006W	VPS27	-0.077	-0.828	-1.732
IYBR161W		-0.353	-0.54	-0.9
IYNL167C		-0.361	-0.502	-0.843
YDL147W	RPN5	-0.385	-0.561	-0.871
IYHR070W		-0.436	-0.599	-0.834
YOR094W	ARF3	-0.368	-0.514	-0.757
YKL158W		-0.563	-0.554	-1.109
IYLR162W-A		-0.57	-0.704	-1.955
IYLR162W-0		-0.634	-0.719	-1.63
INTQ0185A		-0.508	-0.495	-1.307
YER162C	RAD4	-1.411	-0.714	-0.989
YHR210C		-1.032	-0.527	-0.993
YDR323C	PEP7	-0.588	-0.312	-0.52
IYDR189W		-0.551	-0.305	-0.518
YLL010C	PSR1	-0.678	-0.517	-0.709
YBL076C	ILS1	-0.629	-0.427	-0.662
IYDL102W		-2.243	-1.462	-1.996
YDR355C		-0.681	-0.479	-0.559
YHR143W	DSE2	-0.533	-0.214	-0.559
IYCR104W		-0.54	-0.18	-0.578
IYHR204W		-0.548	-0.245	-0.639
YMR076C	PDS5	-0.583	-0.195	-0.521
YDR137W	RGP1	-0.528	-0.349	-0.708
YEL021W	URA3	-0.835	-0.547	-0.995
YBR179C	FZO1	-0.53	-0.329	-0.645
IYNL281W		0.626	0.17	0.546

IYHR164C		0.889	0.209	1.051
YGR243W		0.603	0.075	1.034
YBR267W	REI1	-0.108	0.983	0.645
YCR010C	ADY2	-0.132	1.057	0.549
YOR211C	MGM1	-0.155	0.807	0.912
YHR179W	OYE2	0.551	0.941	0.326
YOR065W	CYT1	0.599	0.965	0.354
YPL006W	NCR1	0.574	1.08	0.438
YGL010W		0.616	0.986	0.441
IYGL177W-2		0.61	1.028	0.499
YJR128W		1.321	1.725	1.197
YDL223C	HBT1	0.757	1.008	0.629
YBL109W		0.885	1.197	0.765
YMR266W		0.595	0.886	0.543
IYKR042W		0.573	0.823	0.47
YGR121C	MEP1	0.977	1.433	0.838
IYIL034C		0.637	1.109	0.729
YJR020W		0.623	1.061	0.658
YBR067C	TIP1	1.816	3.038	1.844
IYOR387C-1		0.528	0.839	0.536
YMR169C	ALD3	0.523	0.691	0.339
YGR041W	BUD9	0.538	0.7	0.353
YJL116C	NCA3	2.322	2.971	1.594
ITN(GUU)G		0.82	1.075	0.46
IYDL204W		0.609	0.789	0.677
YDR533C	HSP31	0.497	0.571	0.533
YJR153W	PGU1	0.698	0.843	0.744
YKL224C		0.569	0.766	0.584
YGL104C	VPS73	0.46	0.588	0.622
YCR104W	PAU3	0.596	0.787	0.755
YAL068C		0.778	0.761	0.695
YHL045W		1.325	1.515	1.048
YOL086C	ADH1	0.751	1.762	0.719
YHL043W	ECM34	0.436	1.16	0.55
YDR050C	TPI1	0.475	1.17	0.515
YDL218W		0.783	2.035	0.904
YPL092W	SSU1	0.329	1.241	0.661
IYHR028C		0.192	1.037	1.097
IYCL040W		0.372	1	0.613
YKR009C	FOX2	0.484	1.331	0.916
IYLL016W		0.345	1.353	0.898
YOR374W	ALD4	0.347	1.393	1.081
IYKL101W		0.309	0.751	0.66
YIR038C	GTT1	0.297	0.804	0.641
YNR002C	FUN34	0.371	0.971	0.796
YBR068C	BAP2	0.345	1.017	0.936
IYNL056W		0.362	0.715	0.513
YPL171C	OYE3	0.445	0.694	0.57
YDR256C	CTA1	0.686	1.206	0.978
YER177W	BMH1	0.311	0.516	0.602
IYNL072W		0.626	1.076	1.198

IYGR095C		0.471	0.701	0.809
YJR019C	TES1	0.56	0.966	0.89
IYBRCDELTA18		0.382	0.615	0.6
YDR544C		0.505	0.776	0.781
IYOL148C		0.223	0.568	0.629
YDL171C	GLT1	0.21	0.578	0.6
YLL025W		0.285	0.612	0.661
YCR103C		0.664	0.694	0.836
IYPL224C		0.945	0.94	1.259
IYLR461W-1		0.436	0.584	0.868
YPL048W	CAM1	0.732	0.976	1.547
IYOR002W		0.61	0.541	0.965
YGR158C	MTR3	0.264	0.525	0.894
YGL262W		0.661	0.369	0.578
IYOR390W		0.823	0.397	0.613
YCL006C		0.848	0.573	0.679
YOL154W	ZPS1	1.133	0.859	0.964
IYFR052W		0.723	0.599	0.463
IYBR203W		0.509	0.542	-0.042
IYNLCDELTA1-2		0.669	0.842	-0.166
YBR212W	NGR1	0.511	0.75	-0.064
YER086W	ILV1	0.536	0.966	-0.053
YAR068W		0.612	0.965	0.237
YDR233C	RTN1	0.631	0.807	0.109
YLR044C	PDC1	0.772	1.044	0.164
IYDR170W-A-0		0.522	0.609	0.071
YGL040C	HEM2	0.54	0.551	0.033
YBR158W	AMN1	0.557	0.548	0.07
IYLL067C-1		0.97	0.962	0.11
IYJL117W		1.4	1.084	0.475
YHR217C		0.765	0.704	0.221
YNL274C		0.924	1.017	0.352
YDL140C	RPO21	0.504	1.143	0.342
YFL039C		0.543	1.298	0.326
YPL036W	PMA2	0.798	2.224	0.614
YCR013C		0.987	2.779	0.68
YGR284C	ERV29	0.562	1.547	0.337
YBL083C		0.601	1.584	0.365
YFL030W	AGX1	0.544	1.527	0.139
YBL099W	ATP1	0.541	1.586	0.168
IYPR015C		0.587	1.613	0.187
YGR279C	SCW4	0.799	1.973	0.238
IYEL047C		0.621	1.349	0.252
YJR117W	STE24	0.536	1.314	0.231
YCR012W	PGK1	0.984	2.341	0.437
YDR012W	RPL4B	0.62	1.744	0.313
YHR174W	ENO2	0.964	2.635	0.47
YHL016C	DUR3	0.628	1.642	0.312
YJL052W	TDH1	0.791	2.102	0.373
YJL026W	RNR2	0.603	1.673	0.249
YBR287W		0.589	2.182	0.361

YGR254W	ENO1	0.616	2.196	0.334
YKR042W	UTH1	0.53	1.564	0.09
YEL046C	GLY1	0.617	1.961	0.044
YBR196C	PGI1	0.513	1.829	0.078
YBR031W	RPL4A	0.716	2.339	0.138
YBR221C	PDB1	0.553	1.486	-0.073
YMR015C	ERG5	0.786	1.778	-0.146
YGR088W	CTT1	0.573	1.264	-0.03
YLR056W	ERG3	0.704	1.55	-0.051
YBL113C		0.542	1.175	0.152
YNL160W	YGP1	0.932	1.872	0.177
YOR383C	FIT3	0.71	1.482	0.07
YOR002W	ALG6	0.541	1.219	0.074
YBR222C	PCS60	0.796	1.893	0.086
IYJL091C		1.985	0.964	0.251

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